SEXUAL CONFLICT OVER MATING IN *LYGAEUS* SEED BUGS

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Thesis Abstract

Sexual conflict has been proposed to be important for evolution, and is often implicated in population divergence and speciation through sexually antagonistic co-evolution (SAC). However, empirical tests of these ideas on field populations are few. How sexual conflict, and SAC, operates in the wild, remains an important unanswered question if we are to fully understand the role of sexual conflict in evolution in nature. Here, I studied sexual conflict over mating in the seed feeding bugs *Lygaeus equestris* and *Lygaeus simulans*. Firstly, I show that laboratory adapted populations of *L. equestris* that differ in the magnitude of sexual conflict also differ in aspects of their reproductive development and mating propensity, with the population displaying greater conflict load also mating more readily. Study of female receptivity to mating as an evolvable trait, that could be involved in conflict over mating, revealed moderate to low heritability at two age groups. To better understand variation in the expression of sexual conflict in the wild, field caught populations of *L. equestris*, ranging across its distribution, and also of its sister species, *L. simulans*, were assayed for the magnitude of sexual conflict over mating in common garden laboratory experiments. High female mating costs were apparent across the populations, but the magnitude of these costs did not vary. No consistent patterns of mating costs and life history variation were found however, arguing against close links between mating costs and life-history. Finally, I investigated whether populations displaying sexual conflict over mating have begun to diverge, and evolve reproductive isolation. I found no evidence of reproductive isolation, or variation in mating propensity, between populations of *L. equestris* when crossed in reciprocal no-choice mating trials. However, *L. equestris* and *L. simulans* did show pre-zygotic reproductive isolation albeit with asymmetries between the reciprocal crosses (*L. simulans* males were able to mate *L. equestris* females, but male *L. equestris* were largely unable to mate *L. simulans* females). As expected for close taxa that perhaps have not been diverged for long, pre-zygotic isolation was perhaps stronger than post-zygotic isolation, as F2 offspring were generated by some of the inter-specific crosses: gene flow can therefore occur between these species contrary to previous studies. My data suggest that sexual conflict over mating may reduce the likelihood of speciation through the evolution of male persistence, as well as promote it through population divergence.

Declaration

I declare that, unless stated otherwise, all the work presented in this thesis is my own, and performed by me. I designed, and performed, all the experiments below, however scientific research thrives when it is collaborative in nature and all of the chapters in this thesis were improved by valuable contributions of the people listed below, to whom I am exceedingly grateful.

Chapter 2 – The review of the study system was helped by discussions with Prof. Christer Solbreck in Uppsala, Sweden, who presented me with my first contact with *Lygaeus equestris* in the field, and allowed access to literature.

Chapter 3 – David Shuker originally conceived the experiment, and collaborated with the statistical analysis. I designed and performed the experiments. Sahran Higgins assisted in the laboratory for the quantitative genetics experiment. Alastair Wilson provided guidance with the quantitative genetics analysis of female receptivity.

Chapter 4 – These experiments were conceived by discussions with David Shuker and me. I designed and performed the experiments. Christer Solbreck kindly allowed me to sample populations from his field sites in Sweden, and advised on potential field sties in Italy. David Shuker sampled *Lygaeus simulans* from Tuscany in Italy. David Shuker and Tom Little provided advice and discussion on the statistical analysis.

Chapter 5 – These experiments were conceived by discussions with David Shuker, Tom Little and myself. I designed and performed the experiments. Toby Nowlan assisted in the laboratory with the first experiment. Discussions with David Shuker gave helpful guidance and advice on the statistical analyses.

Chapter 6 – David Shuker, Tom Little, and many others provided helpful discussions throughout this project all of which contribute to the ideas in the discussion. Some of the ideas also arose from interesting discussions at the ISBE symposium in Perth 2010.

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Chapter 1

General Introduction

Background

Sexual conflict is defined as a conflict between the evolutionary interests of males and females (Parker 1979). It is predicted to occur whenever the sexes differ in their optimal value of a given trait (Parker 1979; Arnqvist and Rowe 2005; Parker 2006). Although, for internal fertilisers, the sexes must come together to reproduce, they invariably disagree over various aspects of the 'economics' of reproduction (Arnqvist and Rowe 2005). The outward presence and indeed abundance of sexually dimorphic phenotypes, demonstrates sex-specific selection – fundamental to sexual conflict – to be prominent in nature (Andersson 1994; Arnqvist and Rowe 2005). Thus, optimal trait values for one sex are often likely to be sub-optimal for the other sex, resulting in sexual conflict (Arnqvist and Rowe 2005). For internally fertilising species for example, male reproductive success is largely dependent on the number of females inseminated, whilst female reproductive success is limited by the number of viable eggs produced (Bateman 1948; Arnqvist and Nilsson 2000; Simmons 2005). Mating imposes time and energy costs on females (Watson *et al.* 1998), with each successive mating increasing the risk of predation (Rowe 1994), and infection (Thrall *et al.* 2000), and may cause physiological (Chapman *et al.* 1995) and physical harm (Crudgington and Siva-Jothy 2000) to females. Moreover, multiple mating is known to reduce female longevity in many insects (Arnqvist and Nilsson 2000), where just one, or a few, matings is often sufficient to fertilise all of a female's eggs (Arnqvist and Nilsson 2000; Arnqvist and Rowe 2005; Shuker *et al.* 2006). Consequently females are expected to maximise their fitness with low mating rates whilst males should maximise their fitness with high mating rates, resulting in conflict over mating (Arnqvist and Rowe 2005).

Under true monogamy no conflict is expected because a male's fitness equals that of his mate and no conflict over reproductive decisions will ensue (Arnqvist and Rowe 2005). However, the vast majority of sexually reproducing species display some degree of polygamy (reviewed by Arnqvist and Nilsson 2000; Jennions and Petrie 2000), and sexual conflict is thus predicted to be prevalent in nature (Chapman *et al.* 2003; Arnqvist and Rowe 2005; Chapman 2006). Indeed, conflict has been found across the whole spectrum of male-female interactions including courtship, mating, fertilisation and parental investment (reviewed by Chapman *et al.* 2003; Arnqvist and Rowe 2005; Chapman 2006).

Two general forms of sexual conflict are recognised at the genetic level, namely intra-locus conflict and inter-locus conflict (Parker and Partridge 1998; Arnqvist and Rowe

2005). For intra-locus conflict, conflict may be apparent because alleles at the same locus in males and females confer different fitness benefits to the bearer depending on its sex. Inter-locus sexual conflict, on the other hand, describes situations where there is conflict between alleles at different, interacting, loci in the two sexes (Arnqvist and Rowe 2005; Chapman 2006). As the sexes share most of their genome (excluding sex chromosomes), intra-locus sexual conflict may be prominent, particularly as the evolutionary interests of individuals rarely coincide, let alone that of the sexes (different sex roles, Bateman 1948; Trivers 1972).

Strong evidence for intra-locus conflict was found by clever genetic techniques available for *Drosophila melanogaster*, where the fitness consequences of sets of alleles were assayed for males and females sharing an otherwise identical genetic background (Chippindale *et al.* 2001). Although little difference was found in early developmental stages, in adults, alleles beneficial to one sex were largely detrimental to the other (Chippindale *et al.* 2001). One possible implication of intra-locus conflict is that it may hinder adaptive evolution in both sexes, as selection in one sex impedes the adaptive evolution of the other (Parker and Partridge 1998; Chippindale *et al.* 2001). However, intra-locus conflict may only be transient as sex linkage, and sex specific expression of such alleles is likely to result (and is highly apparent in nature, see Arnqvist and Rowe 2005). Such sex specific expression would result in an end to that particular conflict, however conflict may continue via inter-locus conflict (Harano *et al.* 2010). Thus, more study has been directed at inter-locus conflict. This describes a conflict between the sexes over the outcome of interactions between the sexes, and broadly conforms to conflict over aspects of mating and parental investments, and the traits that underpin them (Lessells 2006). This thesis concentrates on sexual conflict over mating.

The underlying importance attributed to sexual conflict, in addition to its expected prevalence, is its inherent potential to drive rapid and divergent evolutionary change via sexually antagonistic co-evolution (SAC, Parker 1979; Holland and Rice 1998; Rice 2000; Arnqvist and Rowe 2005; Lessells 2006). For inter-locus sexual conflict, reciprocal adaptation and counter-adaptation between the sexes will shift the interaction outcome towards the respective optima of one or other sex. SAC can thereby result in complex co-evolutionary dynamics, including cyclical dynamics and irresolvable evolutionary chases (e.g. arms races, Parker 1979; Holland and Rice 1998; Rice 2000; Gavrilets *et al.* 2001; Lessells 2006).

Models of sexual conflict differ to traditional sexual selection models chiefly in that co-evolution occurs not because a male trait is beneficial to and 'preferred' by females (Andersson 1994), but because the male trait increases male fitness at the expense of female fitness, and females then respond to reduce this fitness cost (Parker 1979; Holland and Rice 1998; Gavrilets *et al.* 2001). This situation essentially parallels models of sexual selection whereby females gain direct benefits from mating particular males, either through nutrient donation in the ejaculate, or spermatophores, or through increased parental care (Andersson 1994). However, under sexual conflict the evolution of the female preference is more clearly understood as female avoidance of male induced costs (resistance), rather than gaining benefits from preferred males (reviewed by Chapman *et al.* 2003; Arnqvist and Rowe 2005). Unlike 'Fisherian' type models of sexual selection (including, good genes, sexy sons and honest indicator mechanisms (Andersson 1994), where the male trait reflects (is correlated with) male fitness, a genetic correlation between the trait in males and preference in females, is not required for sexually antagonistic co-evolution to operate (reviewed by Arnqvist and Rowe 2005). Therefore, rather than trait exaggeration in males evolving due to indirect selection on the female preference (where the male trait is genetically linked to the female preference) and vice versa, under sexual conflict models, females are under direct selection to reduce the costs of mating imposed upon them (Andersson 1994; Arnqvist and Rowe 2005).

The quantitative genetic model of Gavrilets et al (2001) supported previous game theory models (e.g. Parker 1979), and verbal models (Holland and Rice 1998) of sexual conflict in predicting co-evolutionary chases between the sexes. Here, the direct costs of mating with manipulative males resulted in increased, costly, female resistance to evolve by means of a shift in the threshold male persistence required to ensure mating. A perpetual arms race between male exaggeration and female resistance then ensued over a range of circumstances (Gavrilets *et al.* 2001). However, Gavrilets et al (2001) constrained the evolutionary response of females to male manipulation (i.e. the female preference function) such that females could only respond to increased male manipulation by increasing the threshold manipulation required by males to facilitate mating. The result of this threshold response by females potentially over-estimates the likelihood of co-evolutionary arms races between the sexes, as females could respond in other ways (Rosenthal and Servedio 1999; Rowe *et al.* 2005). For example, females could evolve insensitivity, or plasticity, to the male trait. The shape of the preference/resistance function could then evolve in addition to the threshold male persistence required for mating (Rowe *et al.* 2005). Models incorporating

female sensitivity to male traits resulted in the prevention of male trait exaggeration, and a continuous chase, or even in its reversal, as female insensitivity to male adaptation renders the male adaptation costly to males without the benefits of increased mating (Arnqvist and Rowe 2005; Rowe *et al.* 2005).

This suggests that arms races between the sexes may not be as common as previously thought. However, these equilibrium models only consider the evolution of one male trait and one corresponding female preference function (Arnqvist and Rowe 2005). Males could respond to female indifference to a male trait (that was previously beneficial to males in inducing mating) by evolving a new manipulative trait that mediates a different pathway in females (Arnqvist and Rowe 2005). Such responses could once more lead to potentially endless chases, or fluctuating, frequency dependent selection, limited only by the number of exploitable pathways in females, and the genetic variability among males to exploit them (Arnqvist and Rowe 2005). Indeed, due to the large number of factors likely to be involved in determining interaction outcomes, co-evolutionary races between males and females may act simultaneously over multiple traits, involving many loci (Parker and Partridge 1998; Gavrilets 2000; Arnqvist and Rowe 2005). As such, distinct populations may evolve along alternative co-evolutionary trajectories, promoting population divergence and reproductive isolation in the wild (Parker and Partridge 1998; Gavrilets 2000; Martin and Hosken 2003; Arnqvist and Rowe 2005).

As discussed above, sexual conflict may not always lead to SAC (Chapman 2006). For SAC to occur there must be both opposing selection (natural or sexual) on traits in males and females, and genetic variation in traits of males and females that underlie the conflict (Chapman 2006). Even where sexual conflict is apparent, the consequences for evolution are uncertain (e.g. Gavrilets and Hayashi 2005). Although conflict could result in continuous chases between the sexes across populations, and eventual allopatric speciation (Holland and Rice 1998; Parker and Partridge 1998; Gavrilets 2000), conflict could also lead to trait diversification within females (Gavrilets and Waxman 2002; Gavrilets and Hayashi 2005). In this situation, resultant female polymorphisms may be maintained by negative frequency dependent selection preventing continuation of the evolutionary chase and so limiting further SAC (Gavrilets and Waxman 2002; Svensson *et al.* 2005; Härdling and Bergsten 2006). This may have occurred, for example, in the damselfly *Ischnura elegans* (Svensson *et al.* 2005), where males appear to be caught between three female morphs. Males form a search image for partners (Van Gossum *et al.* 1999), and common morphs are subsequently harassed more

by males (Fincke 2004), leading to greater mating rates, and rare morph advantage (Svensson *et al.* 2005). However, female diversification could also be followed by male diversification (Gavrilets and Waxman 2002). In this case, divergence in sympatry could potentially result from assortative mating of particular male types with particular female types (Gavrilets and Waxman 2002; Svensson *et al.* 2009), but this would seem unlikely given that males are generally predicted to mate on encountering a female, and thus restricting assortative mating (Parker and Partridge 1998).

As SAC is expected to select for generally persistent and exploitative males, the likelihood of sexual conflict and SAC in promoting population divergence, reproductive isolation, and thus speciation has been questioned (Parker and Partridge 1998). Speciation between allopatric populations on secondary contact has been proposed from sexual conflict, as populations may be likely to evolve along divergent SAC trajectories, such that the respective signal-receptor signals for mating may also have diverged (Arnqvist and Rowe 2005). The duration of allopatry is likely to be important therefore, in determining whether particular mate recognition cues have diverged sufficiently to prevent interbreeding. However, given the 'value of winning' for males is high, in that males are predicted to mate, it may pay males to attempt to mate with any potential mate rather than to forgo the opportunity and continue searching (Parker and Partridge 1998). Thus, SAC may actively restrict population divergence as males would be selected to mate any female, and if any hybrid offspring are at least not of significantly poorer fitness, geneflow between the populations would occur, restricting divergence and promoting introgression of the genomes (Parker and Partridge 1998). Indeed, for divergence to occur on secondary contact assortative mating is necessary, and can only occur if females are able to restrict mating with foreign males. However, if divergent co-evolutionary trajectories have occurred between the respective populations, females may not be able resist mating with the foreign males they have not co-evolved with and developed the appropriate resistance mechanisms. Thus, the instigation of manipulative male traits by sexual conflict, may restrict diversification rather than promoting it through arms races or otherwise, and the consequences of sexual conflict for evolution remain unresolved. Indeed, Gavrilets and Hayashi (2005) show theoretically that at least six types of SAC dynamics can be generated from the same model, depending on initial conditions, including continuous chases, evolution towards an equilibrium (or line of equilibrium), cyclic evolution, diversification in female traits, and diversification in both male and female traits. Only two of these (continuous chases and diversification of both female and male traits) allowed for the possibility of speciation, however, stochastic

perturbations can potentially switch the dynamics from one regime to another, making evolutionary consequences of sexual conflict very difficult to predict (Gavrilets and Hayashi 2005). Overall, the sex-specific patterns of selection needed for sexual conflict may seem abundant due to the different roles of the sexes in reproduction (Bateman 1948; Trivers 1972; Arnqvist and Rowe 2005), but such sex specificity need not be as common, or might be more context dependent than we think (Chapman 2006).

Detecting sexual conflict

Sexual conflict will be hidden if you are looking in the wrong place (reviewed by Arnqvist and Rowe 2005; Chapman 2006). For example, conflict over mating might be played out via many different physiological (e.g. accessory proteins), morphological (genital morphology), behavioural (receptivity to mating), and genetic systems (Arnqvist and Rowe 2005; Chapman 2006). Mating rate may not, therefore, be the actual conflict trait, but rather the context in which conflict occurs (Arnqvist and Rowe 2005). It is thus important to be clear about what the conflict trait actually is, and what it is affecting when attempting to measure conflict and SAC (Chapman 2006). Additionally, due to its very nature, SAC may itself remain hidden or overlooked in studies where male and female traits are observed at just one time point (Arnqvist and Rowe 2005; Chapman 2006).

Even where sexual conflict is apparent and conflict trait values (e.g. male persistence/female resistance to male mating phenotypes) seem to favour one sex, it may be unclear whether this sex is 'winning' an arms race. Other factors resulting from natural selection, such as the organism's life history, ecology, or environment, may also be responsible for the observed pattern (Härdling and Kaitala 2005; Kokko and Rankin 2006; Shuker *et al.* 2006). If natural selection pressures associated with the ecology of the species, or its environment proved to be the overriding factor(s) determining the phenotypic differences, sexual conflict may only have a minor role, and thus need not be invoked to explain evolutionary change. In all likelihood there may be complex interactions between natural and sexual selection processes, such that dissecting the primary cause of observed sexual conflict may prove difficult and unrewarding (Kokko and Rankin 2006; Shuker *et al.* 2006).

The extent to which sexual conflict is involved in determining observed inter-sexual patterns is of great importance if we want to understand its role in evolution in the wild.

Fundamentally the environment will determine how natural selection and sexual selection act on males and females, and the extent to which patterns of selection come into conflict between the sexes (Härdling and Kaitala 2005; Kokko and Rankin 2006). To determine the consequences of sexual conflict for evolution empirically, comprehensive information is required including: (1) the costs and benefits of the conflict trait in both sexes and the associated trait that it is affecting; (2) temporal and spatial (cross population) variance in the trait; and (3) life-history, ecology, and environmental factors influencing the trait (reviewed by Chapman 2006; Kokko and Rankin 2006). With this information we will be better able to attempt to disentangle effects of various selection pressures on various sexual conflicts.

Sexual conflict over mating

Although females tend to have a lower potential reproductive output compared to males, females of the majority of species are known to mate multiply (Arnqvist and Nilsson 2000; Jennions and Petrie 2000). Studies have shown that despite incurring various costs from mating, which may not be insubstantial (Rowe 1994; Chapman *et al.* 1995; Watson *et al.* 1998; Crudgington and Siva-Jothy 2000; Thrall *et al.* 2000), low levels of multiple mating may be beneficial to females, outweighing these costs (Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Simmons 2005; House *et al.* 2008). As such, for any given mating event there may be no conflict between the male-female pair if both gain from the mating. Female benefits include direct benefits which increase the female's fitness (see Arnqvist and Nilsson 2000), and potential genetic benefits from mating multiple males that increase offspring fitness (e.g. Tregenza and Wedell 1998; reviewed by Jennions and Petrie 2000; Zeh and Zeh 2001; Simmons 2005). Therefore, the difference between male and female optima for female mating rate, determining the magnitude of the conflict, largely depends upon the balance between the costs and benefits of additional mating for females.

Female mating costs

Female mating costs are integral to, and a requirement of, sexual conflict over mating (Lessells 2006). Female mating costs are not always apparent for promiscuous species however (Arnqvist and Nilsson 2000; Martin and Hosken 2004; Reguera *et al.* 2004), and can vary substantially among (e.g. Rönn *et al.* 2006), and within species (Shuker *et al.* 2006). Why this should be is not immediately clear. The costs of mating may define how far apart the fitness optima of males and females are (i.e. the conflict load). Furthermore, SAC may

result in male and female optima becoming closer or further away, and if female mate costs map to this process, then we may expect the costs of mating to vary over successive cycles of SAC. For instance, female mating costs may be greater where male persistence has the upper hand over female resistance traits, and vice versa (Rice 2000).

The costs of mating will also be influenced by respective ecological and environmental conditions (Härdling and Kaitala 2005), influencing how far apart male and female trait optima are, and thus the level of conflict. For example, predation pressure influences conflict over mating in water striders (Arnqvist 1997; Arnqvist and Rowe 2005), frogs (Lode *et al.* 2004), and guppies (Magnhagen 1991; Croft *et al.* 2006; Elgee *et al.* 2010). Additionally, the magnitude of the overall conflict over mating will be affected by habitat and/or population structure that influences encounter rates between the sexes (e.g. water striders, Eldakar *et al.* 2009a; Eldakar *et al.* 2010a; seaweed flies, Edward and Gilburn 2007; and guppies, Magellan and Magurran 2006; see also Härdling and Kaitala 2005;) and food availability is known to affect the extent of conflict in *Drosophila melanogaster* (Chapman and Partridge 1996), and *Pieris napi* (Wedell *et al.* 2002b). Field studies of Lepidoptera suggest strong context dependence of sexual conflict, with polyandry being beneficial only when sufficient food is available (Wedell *et al.* 2002b). Therefore, evolutionary responses in males and/or females may result from, or lead to, changes in life-history decisions, with further downstream effects on mating behaviour and reproduction. For instance, female guppies may seek areas of increased predation pressure to avoid male harassment (Croft *et al.* 2006; Magellan and Magurran 2006; Elgee *et al.* 2010).

Receptivity to mating

Female receptivity to mating (usually considered in terms of resistance to male mating attempts) is central to determining whether mating occurs upon encountering a male, and is one way in which females could potentially control mating rates. Receptivity to mating is likely underpinned by a wide array of intrinsic, physiological and neurological factors, as well as having considerable environmental influences (reviewed by Ringo 1996; Torres-Vila *et al.* 1997). For example, polyandry is influenced by female size and nutrition, as well as male quality (including spermataphore size), and the abundance of mates in Lepidoptera (Torres-Vila *et al.* 1997; Torres-Vila *et al.* 2005; Wedell 2005). Pair-bond formation is vital for reproduction in the monogamous prairie vole (*Microtus ochrogaster*). Here, ovarian activity and female receptivity is initiated by prolonged contact with a novel male, and is

mediated by neuroendocrine mechanisms, including the neuropeptide vasopressin (Roberts *et al.* 1998; Young *et al.* 2001; Lim and Young 2004).

Furthermore, mating itself commonly stimulates reproduction and reduces female receptivity (increasing the latency to re-mate), as can the presence of sperm in the spermatheca, and accessory proteins transferred to females in the ejaculate (e.g. anti-aphrodisiacs Gromko *et al.* 1984; Andersson *et al.* 2000; Arnqvist and Nilsson 2000; Wolfner 2009), but see (Chapman *et al.* 1998). The amount or intensity of male courtship can also influence the probability of mating and re-mating, and so males may invest substantially in courtship displays (Ringo 1996; Hunt *et al.* 2004a; Shamble *et al.* 2009; Crudgington *et al.* 2010). These effects highlight female receptivity to mating as a function of both female physiology, and male attempts to manipulate female physiology (see Wolfner 2009) thus setting the stage for further conflict. However, along with benefiting from mating, individual males may also seek to prevent females from re-mating in order to protect his paternity of her future offspring (defensive sperm competition: Simmons 2001; Simmons 2005), and highlights that conflict will be affected by sexual selection. Therefore there is often conflict between the sexes over individual mating events (Arnqvist and Rowe 2005), and a tension between offensive and defensive sperm competition adaptations (Gavrilets and Hayashi 2006).

Empirical evidence for sexual antagonism over mating driving evolution

Empirical evidence for the existence of sexual conflict and the operation of SAC over mating has been most clearly demonstrated in laboratory evolution studies (Holland and Rice 1999; Martin and Hosken 2003; Wigby and Chapman 2004; Stewart *et al.* 2005; Rice *et al.* 2006). For example, Holland and Rice (1999) demonstrated that removal of sexual conflict in *Drosophila melanogaster* populations, by enforced monogamy, resulted in reduced male harm to their mates, reduced female resistance to male harm, and greater net reproductive rate than promiscuous populations. This suggested that male induced harm from promiscuity is costly to females, the population as a whole, and that sexual conflict over mating may be widespread (Holland and Rice 1999). The presence of sexual conflict over mating in *D. melanogaster* is supported by further studies (e.g. Stewart *et al.* 2005; Rice *et al.* 2006). Instead of enforcing monogamy *per se*, Stewart et al (2005) used an artificial selection experiment to evaluate sexual conflict over mating in polygamous laboratory populations. A mutation giving females high resistance against male harassment, and re-mating, repeatedly

spread through replicate populations, demonstrating that the female mating costs alleviated by the increased resistance to mating were greater than potential indirect genetic benefits of mating manipulative males, and thus that sexual conflict over mating was apparent (Stewart *et al.* 2005; Rice *et al.* 2006). In the field, evidence of SAC over mating comes from large comparative studies of male and female phenotypes that are expected to be associated with mating probability (Arnqvist and Rowe 2005). Such inter-specific studies have revealed correlated evolution of armaments between the sexes (correcting for phylogeny), among water striders (Arnqvist and Rowe 2002a; Arnqvist and Rowe 2002b), diving beetles (Bergsten *et al.* 2001; Bergsten and Miller 2007), plant bugs (Tatarnic and Cassis 2010), and even in hermaphroditic land snails (Koene and Schulenburg 2005) reflecting arms races over mating rate (see also Anthes *et al.* 2008).

A proposed evolutionary consequence of sexual conflict, and SAC, is that of reproductive isolation between allopatric populations, resulting from population divergence. Evidence of sexual conflict over mating, and SAC, promoting population divergence and reproductive isolation, has been shown in the dung fly, *Sepsis cynipsea* (Martin and Hosken 2003). Martin and Hosken (2003) found that divergent SAC among laboratory populations of *Sepsis cynipsea* (held under varied levels of sexual conflict) led to increased reproductive isolation among the populations after 35 generations (Martin and Hosken 2003). However, experimental manipulation of sexual conflict has not always been found to promote reproductive isolation among replicate populations in the laboratory, thus questioning the generality of SAC as a driver of intra-specific population divergence (Wigby and Chapman 2006; Bacigalupe *et al.* 2007; Gay *et al.* 2009; Maklakov *et al.* 2010). For example, Bacigalupe *et al* (2007) found no evidence that sexual conflict drove reproductive isolation among populations of *Drosophila pseudoobscura*, subjected to varied levels of sexual conflict. Thus, no support was found for existing theory suggesting that sexual conflict, and indeed greater levels of conflict, may lead to faster divergence of reproductive traits, and increased reproductive isolation among populations (Parker and Partridge 1998; Gavrilets 2000; Gavrilets and Hayashi 2005).

Similarly mixed results, with respect to SAC and its implications for reproductive isolation, have been found with experiments of mating behaviour between allopatric populations. For example, asymmetric mating among allopatric populations has been suggested to be due to the evolution of differential levels of male vigour, and counter balancing (co-evolved) female resistance, across populations of Sonoran desert *Drosophila*

sp, (Markow and Hoccutt 1998), the grasshopper, *Podisma sapporensis* (Sugano and Akimoto 2007), and the jumping spider, *Habronattus pugillis* (Hebets and Maddison 2005). Other studies however, have found little effect of differential SAC among populations on the outcome of inter-population mating interactions, even where sexual conflict is apparent, such as in the water strider, *Gerris gillettei* (Gagnon and Turgeon 2011).

In the seed beetle *Callosobruchus maculatus*, applying life-history selection in conjunction with sexual selection in the laboratory revealed life-history selection to be stronger than sexual selection, derived from sexual conflict (Maklakov *et al.* 2009; Maklakov *et al.* 2010). Females selected for late reproduction showed reduced mating propensity early in life, regardless of being retained under monogamy or polygamy (Maklakov *et al.* 2010). Nevertheless, when replicate lines exposed to the same selection regimes were crossed (allopatric crosses), the mating system had divergent effects on mating depending on the life history selection imposed (Maklakov *et al.* 2010). Mating was greater among polygamous populations selected for early life reproduction, however when selected for late life reproduction, mating was greater amongst monogamous populations (Maklakov *et al.* 2010). The authors argue that this highlights an apparent context dependence of the interaction between life history selection and sexual selection on reproductive divergence (Maklakov *et al.* 2010). This could reflect sexual selection promoting life-history selection, and vice verse, in both instances. For example, there may be an apparent lack of sexual conflict when selecting for early reproduction in polygamy, as both males and females will be selected to mate. On the other hand, selection for late life reproduction may promote female resistance to mating, as typified under sexual conflict, and act to reduce mating across populations enhancing population divergence. Indeed, the utility of studying inter-population crosses in common garden laboratory experiments to examine sexual conflict over mating has been questioned (Long *et al.* 2006), as SAC may not be expected to produce any particular pattern in inter-population mating crosses (Chapman *et al.* 2003; Long *et al.* 2006; Tregenza *et al.* 2006).

Due to the large amount of information required to test for the operation of SAC in the wild, and to disentangle it from other processes of sexual (Eberhard 2004) and natural selection from local adaptation (Chapman 2006; Kokko and Rankin 2006; Panhuis *et al.* 2006; Maklakov *et al.* 2010), comprehensive studies remain limited in number (Arnqvist and Rowe 2005; Chapman 2006). Thus, the general importance of sexual conflict and SAC as a driver of evolution in the field remains relatively unclear (Chapman 2006; Gagnon and

Turgeon 2011). In order to address the relative importance of sexual conflict for evolution in terms of female mating costs, both field and laboratory studies are needed. They are necessary to determine how life-history and ecological parameters co-vary with costs. Natural variation in female mating costs across populations should thus be explored to determine the relative importance of sexual conflict and SAC in driving these patterns compared with ecological and life-history factors.

Aims and Objectives

The aim of this thesis is to explore sexual conflict over mating rate in intra-specific and inter-specific studies using the seed bugs, *Lygaeus equestris*, and *Lygaeus simulans***.** These species are highly promiscuous, yet females incur high mating costs that vary between populations (Sillén-Tullberg 1981; Solbreck *et al.* 1989; Tadler *et al.* 1999; Shuker *et al.* 2006). The large geographic range of the bugs ensures that populations differ in the ecological conditions experienced and in many life-history traits (Solbreck *et al.* 1989; Shuker *et al.* 2006). Thus, these species present an opportunity to explore conflicts over mating in populations varying in life history traits and their ecological context. I explore patterns of male and female receptivity to mating among populations known to vary in the intensity of sexual conflict experienced in the laboratory. Additionally, I use natural variation among populations to test how female mating costs vary across their geographic distribution, and how these sexual conflicts over mating associate with life-history and ecological differences. I also investigate reproductive isolation within and between populations of the two species to explore whether these (presumably largely) allopatric populations have diverged reproductively and how this may relate to varying sexual conflict and life history differences respectively.

Thesis Outline and Chapter Aims

Chapter 2: The Study System. The next chapter outlines the ecology of *Lygaeus* seed bugs, used in the experiments to follow. I also describe the general husbandry methods used in the laboratory in culturing the two species.

Chapter 3: Receptivity to mating and reproductive development in the seed bug *Lygaeus equestris.* Based on evidence from a previous study, I tested whether, and how, populations that vary in the level of sexual conflict also vary in their respective aspects of reproductive development and primary receptivity to mating. I found that the population displaying greater sexual conflict in terms of female mating costs mated more readily, and females of this population developed faster. Additionally, I tested for and found low heritability for female receptivity in a quantitative genetic experiment using one of the populations.

Chapter 4: Variation in conflict over mating within and between two closely related species of *Lygaeus* seed bugs. I assess geographic variation in the extent of sexual conflict in terms of female mating costs, and life history traits across freshly sampled populations of *Lygaeus equestris* and *Lygaeus simulans* in common garden laboratory experiments. I show that large mating costs are apparent among these populations, and that life histories can vary across populations. However, no general or consistent pattern emerges, suggesting that the causes and consequences of sexual conflict may be somewhat independent of life-history evolution.

Chapter 5: Reproductive isolation within- and between- species characterised by a sexual conflict over mating. I show that populations displaying sexual conflict over mating, and differing in their life histories, show no reproductive isolation or population divergence among populations of the same species sampled from across their distribution in the field. Asymmetric pre-mating isolation was found between the two species however, and is discussed.

Chapter 6: General Discussion. I discuss the importance of sexual conflict relative to other ecological selection pressures in promoting evolutionary divergence in the field, and

highlight interesting areas of research and directions of enquiry for sexual conflict that require our attention.

Chapter 2

The Study System

Lygaeus Ecology

The study organisms used in this thesis were the Pentatomorpha Heteroptera, *Lygaeus equestris* and *Lygaeus simulans*, from the Lygaeidae family. *Lygaeus simulans* (Hemiptera: Lygaeidae, Deckert, 1985) can be distinguished from *Lygaeus equestris* (Linnaeus, 1758,) according to the morphology of the antennae base, and male parameres (Deckert 1985; Péricart 1998, see Figure 2). Very little differences in the biology and behaviour of the two species are known (Deckert 1985; Tadler *et al.* 1999). Thus, as most of the studies have been on *L. equestris*, and the distinction between the two species by the taxonomic study of Deckert (1985) has only been made relatively recently, this chapter necessarily focuses on the ecology of *Lygaeus equestris*, although due to their close relatedness, much will likely apply to *L. simulans* also (Tadler *et al.* 1999).

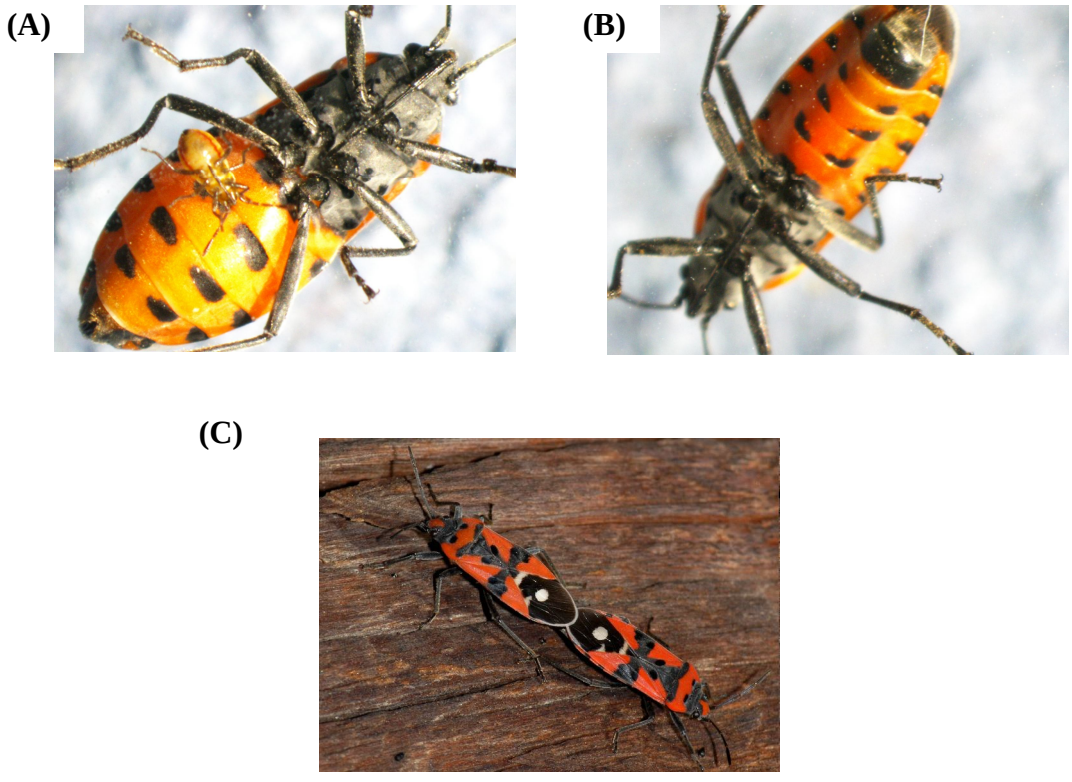


Figure 1. Ventral image of sexually mature female (A) and male (B) *Lygaeus equestris* bugs respectively, and dorsal image of a mating pair (C). Females are characterised by an enlarged abdomen and are larger than males. Males are smaller and narrower with a genital capsule that houses the parameres (genital claspers) and large intromittent organ.

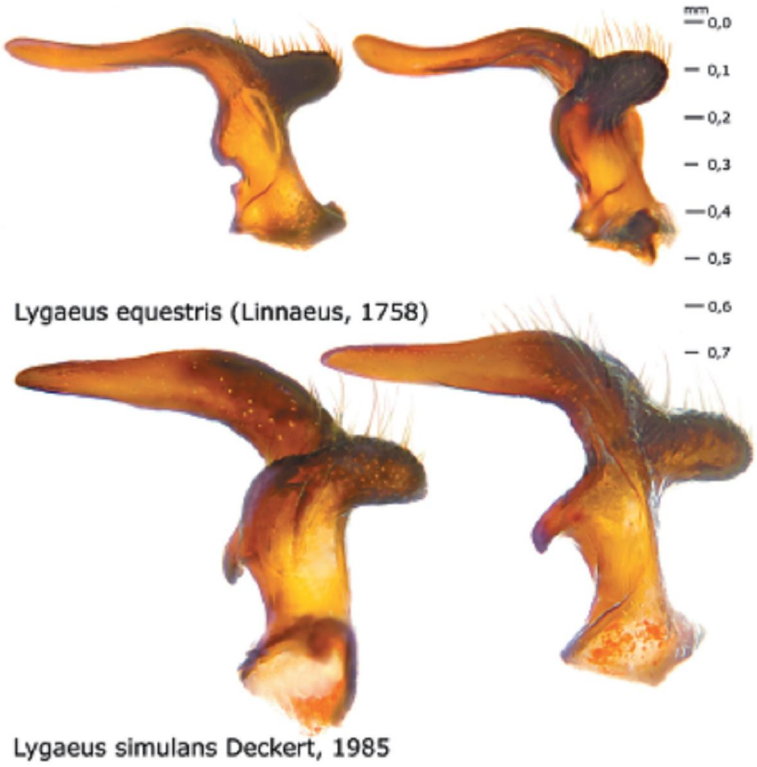


Figure 2. Morphology of male parameres (genital claspers) in *L. equestris* and *L. simulans*. *L. equestris* is distinguishable by the small, semi-circular notch at the base of the parameres. Image adapted from Rabitsch and Deckert (2007).

Distribution

Lygaeus equestris is a widely distributed seed predator occurring across much of Europe, from Spain to Russia (Slater 1964; Winkler and Kerzhner 1977; Deckert 1985; Solbreck *et al.* 1989; Péricart 1998). It feeds on the flower ovules and seeds of many plant species, including various composites and oleanders (Solbreck and Kugelberg 1972; Kugelberg 1973c; Solbreck *et al.* 1989). Its preferred host-plant through much of Europe, however, is *Vincetoxicum hirundinaria* (Gentianales: Asclepiadaceae, Kugelberg 1974; Kugelberg 1977)

upon which it performs better (e.g. higher fecundity, Kugelberg 1973b; Kugelberg 1977), and with which it is most commonly associated in Northern and Central Europe (Solbreck *et al.* 1989). *Lygaeus simulans* has not been observed in Scandinavia (Deckert 1985; Solbreck and Sillén-Tullberg 1990). Both species are aposematic, displaying a red and black warning colouration (Sillén-Tullberg *et al.* 1982; Sillén-Tullberg 1985a; Tullberg *et al.* 2000, Figure 1). Predation, and parasitism, of *L. equestris* has not been observed among Swedish populations (Solbreck *et al.* 1989; Solbreck and Sillén-Tullberg 1990), however attack by tachinid flies is apparent in southern regions (Solbreck *et al.* 1989).

Life-cycle

Most *L. equestris* populations are assumed to be univoltine (producing one offspring generation per year), which is typical for insects of northern latitudes (Solbreck and Sillén-Tullberg 1981). This is facilitated by the induction of reproductive diapause, a seasonal dormancy that allows adults to survive through winter and reproduce the following year. Reproductive diapause is primarily determined by photoperiod, being triggered by the reduced day lengths and temperatures that precede winter (Solbreck and Sillén-Tullberg 1981; Sillén-Tullberg 1984). Diapause induction can be avoided in all *L. equestris* populations, therefore, if conditions allow (i.e. where photoperiod and temperatures do not fall below critical thresholds). The critical photoperiod for diapause induction in *L. equestris* declines with latitude, across its distribution, in line with apparent photoperiods (Solbreck and Sillén-Tullberg 1981). This cline in critical photoperiod affecting diapause, together with the higher temperatures experienced in southern regions, suggests that bi-voltinism (two generations per year) may be more likely to occur amongst southern populations (Solbreck *et al.* 1989). However, even at its North-Western range limit, *L. equestris* can occasionally produce a partial second generation in unusually long, sunny, summers (Solbreck and Sillén-Tullberg 1981; Solbreck 1991).

Two distinct niches are required for *Lygaeus equestris* bugs to survive: hibernation sites for overwintering as adults (Figure 4), and breeding sites at host-plants to feed and reproduce (Figure 3). Thus, bugs display two migratory flight periods per year. In late spring/early summer adult bugs migrate en masse from over-wintering sites (crevices in sun-exposed rocks and walls) to host-plant patches where they reproduce. Bugs may fly substantial distances, up to several kilometres, over many days to find suitable host-plants (Solbreck 1976; Solbreck and Sillén-Tullberg 1990). With the onset of autumn the latest

generation of adults, having built up fat reserves and entered reproductive diapause, migrate to crevices in rocks, and walls of buildings, to over-winter (Solbreck 1976; Solbreck and Sillén-Tullberg 1981). *Lygaeus equestris* congregate at high densities at their hibernation sites, and are exposed to high stochastic mortality rates (Solbreck and Sillén-Tullberg 1990). Densities at host-plant resources in summer are much lower as host plants tend to be patchily distributed (Solbreck and Sillén-Tullberg 1990; Tullberg *et al.* 2000), and can fluctuate greatly temporally in accordance with host plant seed production (Sillén-Tullberg and Solbreck 1990; Solbreck and Sillén-Tullberg 1990).

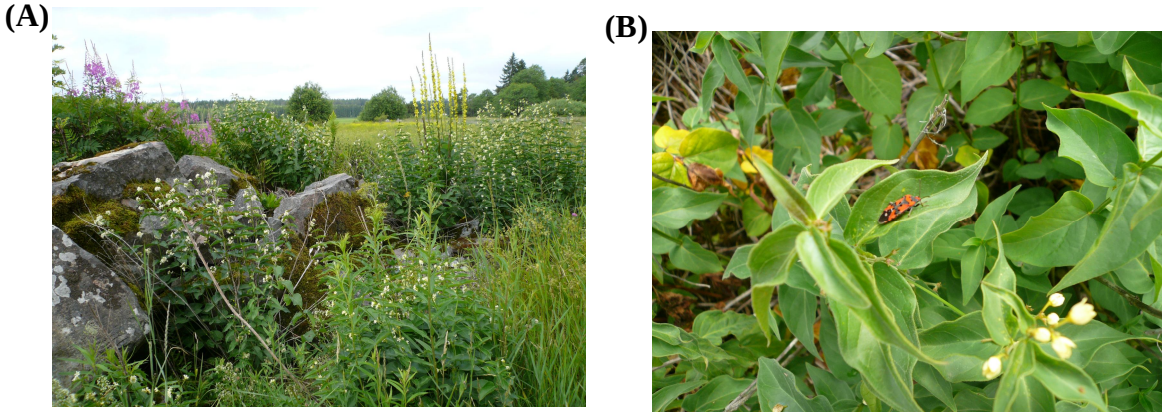


Figure 3. (A) Field site patch of the host plant *Vincetoxicum hirrundinaria*, and (B) dorsal image of *L. equestris*

Lygaeus equestris and *L. simulans* are both highly promiscuous species, with both sexes mating multiply throughout adult life (Solbreck 1971; Solbreck 1972; Sillén-Tullberg 1981; Tadler 1999; Shuker *et al.* 2006). Laboratory observations by Kugelberg (1973b) showed that females may copulate over 40 times in their lifetime. Individual females can oviposit more than 20 egg batches, totalling over 500 eggs when reared on *Helianthus* (sunflower) seeds in the laboratory (Kugelberg 1973b). No direct field polyandry estimates are known, yet they may be high with approximately 60% or more of adults observed to be mating at any one time (Solbreck 1972). However, there may be large population variation in life-history characters across the geographic range of both species, as shown for *L. equestris* (Solbreck *et al.* 1989; Shuker *et al.* 2006). Shuker et al (2006) found that the fecundity of once mated females differed between populations of *L. equestris*, with an average of 150 and 300 eggs produced respectively. Copulation duration also varied among these populations,

with an average of 180 minutes in one population, and 320 minutes in another (Shuker *et al.* 2006).

Mating first occurs at hibernation sites prior to the spring migration and continues post-migration throughout the summer, between oviposition events, until death (Solbreck 1972; Sillén-Tullberg 1981). Typically, mating does not occur in overwintering adults until the end of the hibernation period, and overwintering females exhibit immature ovaries until the following spring migration period (Solbreck 1972). Mating is not essential for ovary maturation and oviposition in *L. equestris*, but it may stimulate reproductive development as is common in insects (Kugelberg 1973b). This stimulatory effect of mating on female reproductive development is likely to account for the finding that mating can also act as a facultative mechanism for limiting diapause induction at threshold conditions where diapause is otherwise induced (Sillén-Tullberg 1984). Following mating, females dig and oviposit eggs in clutches a few centimetres below the soil surface or in the leaf litter around host plants (Sillén-Tullberg 1981; Solbreck and Sillén-Tullberg 1990). They display a long oviposition period, peaking in mid-late June in Sweden, which corresponds to the main flowering period of its favoured host *V. hirundinaria* in this area. Oviposition continues, along with mating, for a month or more until death (Solbreck 1972; Solbreck 1995). Subsequently nymphs and new generation adults also appear over a prolonged period, with the number of young adults peaking around mid August in Sweden (Solbreck and Kugelberg 1972; Solbreck 1995). However, these timings may vary considerably both spatially and temporally due to their wide geographic distribution and varying microclimate conditions.

Figure 4. Sun exposed south facing hibernation site of *L. equestris* in Morga, Sweden.

In the laboratory, at 29°C and a 18:6 L:D cycle, the egg to adult generation time is four to five weeks for *L. equestris* on de-husked *Helianthus* (sunflower) seeds (Shuker *et al.* 2006), and slightly longer on intact seeds (Kugelberg 1973c). The offspring develop through 5 instar stages during this time (Kugelberg 1973c), and following eclosion, adult bugs can live for up to 2-3 months under these conditions (Shuker *et al.* 2006). The development and reproductive performance of *L. equestris* varies substantially depending on the host plant reared upon, but sunflower (*Helianthus*) seeds are a good, readily available, surrogate food resource in the laboratory (see Kugelberg 1973b; Kugelberg 1973c).

Copulation and courtship

No overt pre-mating courtship is displayed in either *L. equestris* (Solbreck 1972; Sillén-Tullberg 1981) or *L. simulans* (Tadler *et al.* 1999). Mating instead consists of a short pre-copulatory struggle, where males attempt to coerce females to mate, followed by copulation for successful males. In the pre-copulatory struggle, males grasp and mount females dorsally, rotating their abdomen under the female, and attempting to insert their aedeagus into the females' bursa copulatrix (Sillén-Tullberg 1981; Tadler *et al.* 1999). A stable end-to-end position is then established between the pair (i.e. between the female's ovipositor and the genital capsule and parameres of the male), after which copulation takes place (Tadler *et al.* 1999). The pair move around and feed in this position, the course directed by the female (Sillén-Tullberg 1981, Figure 1C). Gonad morphology is complex in Lygaeid bugs (Bonhag and Wick 1953; Tadler 1999; Tadler *et al.* 1999; Micholitsch *et al.* 2000; Huber *et al.* 2007). Using *Lygaeus simulans*, Tadler and colleagues (1999) showed that following genital engagement, the male performs intricate genital movements to successfully inseminate the female. The male's genitalia rotates inside the female, with successful insemination requiring the processus gonopori to be inserted into the receptaculi seminis (Figure 5, see also Tadler 1999; Gschwentner and Tadler 2000; Micholitsch *et al.* 2000; Huber *et al.* 2007). A recent study by Chiang (2010) however, suggests an alternative route for insemination in Lygaeidae, whereby the insemination duct is separate to the spermathecal duct, and a medial tube within the spermathecal duct, which may aid sperm to travel directly from the spermatheca to the common oviduct. Such a duct was found in the conifer seed bug, *Leptoglossus occidentalis* (Heidemann), the milkweed bug, *Oncopeltus fasciatus* (Dallas), and the box elder bug, *Leptocoris trivittatus* (Say)(Chiang 2010). We do not yet know if this structure is apparent in *L. equestris* or *L. simulans*, and this requires further study, however,

its presence would likely add to the potential for cryptic female choice among females (Chiang 2010).

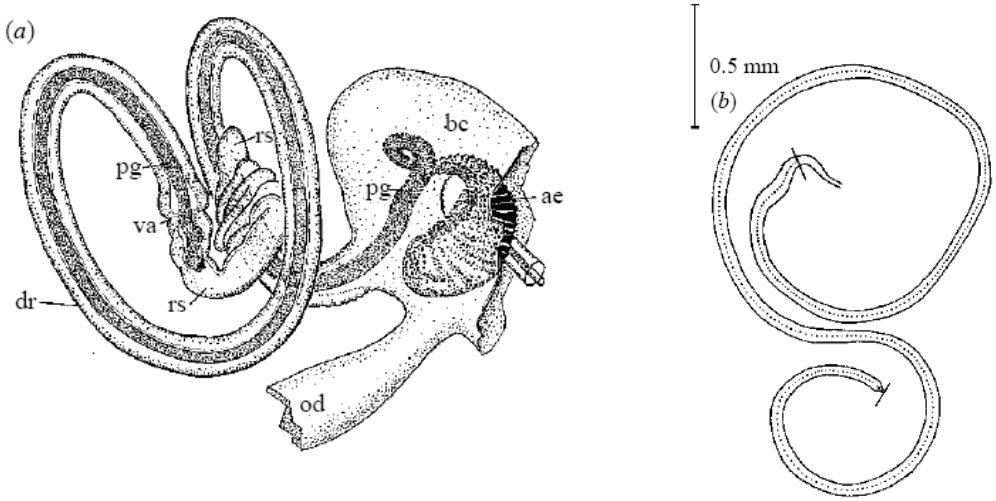


Figure 5. Genital morphology of *Lygaeus simulans*. (a) Male processus introduced into the female receptaculum. The drawing was made from a freeze-fixed preparation. Female parts: bc, bursa copulatrix; dr, ductus receptaculi; od, oviduct; rs, receptaculum seminis; va, valve. Male parts: ae, aedeagus; pg, processus gonopori. (b) Whole-mount preparation of the processus for length measurements (along the dotted line). Adapted from Tadler (1999).

Copulation duration varies dramatically up to, and over, 24 hours in *Lygaeus equestris* (Kugelberg 1973b; Sillén-Tullberg 1981; Shuker *et al.* 2006). Sperm transfer occurs primarily within the first hour or so of copulation (Sillén-Tullberg 1981). Long copulation durations have been suggested to represent copulatory mate guarding by males (Sillén-Tullberg 1981; Shuker *et al.* 2006) as *L. equestris* display a high level of last male sperm precedence (up to 90%) and long copulations do not increase female fecundity (Sillén-Tullberg 1981). However, it is not clear who is in control of copulation duration. Females may copulate with another male immediately after separating from the previous male irrespective of copulation duration (Sillén-Tullberg 1981). Moreover, the behaviour of bugs during copulation is indicative of conflict over mating and mating duration. During copulation females display a side-to-side rocking behaviour (Sillén-Tullberg 1985b) which is prevalent across Lygaeidae (Walker 1979). Rocking in *L equestris* has been interpreted as a copulation termination mechanism by which females either signal to males to terminate copulation, or forcibly loosen the male's grip, allowing the pair to separate (Sillén-Tullberg 1985b). It is more frequent in gravid females, who oviposit soon after separation, than in young females. However, rocking behaviour is observed in virgin and non-gravid females,

albeit to a lesser degree (Sillén-Tullberg 1985b). This may be due to the bug's need to separate in order to defecate, which is inhibited by copulation (Sillén-Tullberg 1985b). As well as female rocking behaviour, *L. equestris* males rhythmically and gently tap the female's abdomen with their legs during copulation. This 'copulatory courtship' behaviour is common in insects and spiders (Eberhard 1994). It is generally assumed to function as a mechanism of influencing cryptic female choice in favour of the performing male (Eberhard 1994), although specific tests of this function are rather limited (e.g. Shuker *et al.* 2002).

Mating costs

Despite the high levels of polyandry exhibited in *L. equestris*, multiple mating is costly to females (Sillén-Tullberg 1981; Shuker *et al.* 2006). For example, when reared with three males for their entire life (three:one male biased sex ratio), female lifespan was reduced by approximately 50%, and fecundity by approximately 60%, of the lifespan and fecundity, respectively, of females retained with a single male (Shuker *et al.* 2006). An earlier study by Sillén-Tullberg (1981) found similarly high mating costs. Females of the majority of species mate multiply, and both direct (material) and indirect (genetic) benefits have been proposed to explain the fitness benefits of polyandry to females (reviewed by Arnqvist and Nilsson 2000; Simmons 2005). Indeed, low levels of polyandry have been shown to increase fitness in many species, and may well benefit *L. equestris* females. For example, Shuker et al (2006) found evidence of sperm depletion in *L. equestris* obtained from Sicily, thus multiple mating may function to avoid this and/or obtain males of higher genetic quality or compatibility.

Nevertheless such high mating costs, resulting from mating itself, should lead to selection against high mating rates and thus for increased resistance to male manipulation. Females may be able resist male mating attempts by making it difficult for the male to curl his abdomen around and achieve intromission, leading to the pre-copulatory struggle. The extent of this struggle is unclear though. Tadler *et al* (1999) found approximately 30% of male mating attempts failed in this way in *L. simulans*. Indeed, even when pairs copulated sperm transfer failures appear to be common, with only 60% of copulations leading to insemination (Tadler *et al.* 1999; Gschwentner and Tadler 2000; Micholitsch *et al.* 2000). Thus, females may well be able to bias paternity, via mate choice and cryptic female choice, more than previously thought (see also Chiang 2010).

Further to the substantial mating costs apparent in *L. equestris*, the magnitudes of these costs differ among the populations tested (Shuker *et al.* 2006). There are many ecological and life-history differences among these populations that may contribute to mating costs. For example, bivoltinism may be more common in southern populations (Solbreck *et al.* 1989), thus early age reproduction may be favoured even if it reduces late life fecundity. The primary host plant *V. hirundinaria* does not occur in Sicily and so *L. equestris* populations occur on other composites and oleanders here (Solbreck *et al.* 1989). The relative nutritional value of host-plants may vary considerably and is known to affect *L. equestris* performance (Kugelberg 1973b; Kugelberg 1973c). This is likely to place an important restraint on, and demand, over energy allocation decisions. Additionally, northern populations of *L. equestris*, such as exist in Sweden, have no recorded predators to contend with, whilst Sicilian populations are exposed to high parasitoid loads (Solbreck *et al.* 1989). Thus the relative cost of reproduction is likely to differ. Indeed this difference in predator pressure is thought to be responsible for the smaller size of bugs in Sicily relative to other populations (Solbreck *et al.* 1989; Shuker *et al.* 2006). Other notable differences include fecundity and clutch size (Shuker *et al.* 2006). Interestingly, much of the phenotypic differences among populations occur through Italy, with little differences among bugs from central and northern Europe (Solbreck *et al.* 1989), suggesting patterns of interesting phylogeography. Such variation in ecological and life-history variables could well have large consequences for the extent of mating costs across populations. Thus, together these two species of *Lygaeus* provide an opportunity to study intra- and inter-specific variation in sexual conflict over mating.

General methods

Animal husbandry

All *Lygaeus* used in these experiments were maintained in large populations, with over-lapping generations, in plastic containers (culture cages measuring 20cm x 10cm x 8cm) containing organic sunflower (*Helianthus*) seeds (*Goodness Direct, Northampton UK*) to a depth of approximately 2cm (500g), and two water tubes (universal tubes filled with carbon filtered water and a cotton wool bung, see Figure 6). A piece of cotton wool was also placed in each cage as a substrate for bugs to hide in. Between four to six population cages were maintained simultaneously for each population. New cages were set up from adults and

nymphs of all ages ($N \approx$ 30-40 individuals) sampled from existing cages. Culture cages were thus staggered in age, providing a constant supply of nymphs and adults. Population cages were discarded at six to eight weeks old. Water tubes were replaced regularly (typically weekly) ensuring a constant supply of water. Culture cages were originally maintained at 29°C, and a 18:6 L:D light regime for the two laboratory adapted *L. equestris* populations. These populations were derived from wild populations sampled from the Dolomite region of Northern Italy (the Dolomite population, sampled in 2004 by Drs David Shuker and Sue Healy), and from Sicily in Southern Italy (originally cultured in Sweden before being established in the laboratory at the University of Leeds in 1996 by Prof. Nina Wedell- the "Leeds" population; Shuker *et al.* 2006).

For Chapters 4 and 5, freshly sampled field populations were collected from across their geographic distribution (Sweden to Italy, by Gethin Evans and David Shuker) and maintained in common garden laboratory conditions where all populations were then retained in continuous culture at 29°C, and a 22:2 L:D light regime (to prevent reproductive diapause induction throughout all populations). Populations were retained in isolation and were crossed in experiments of Chapter 5. All bugs were retained in one incubator, where possible, but extended to two incubators, (a culturing incubator, and an experimental incubator) for the later experiments that used more populations (see Figure 6).

Figure 6. Laboratory setup of *Lygaeus* populations. Containers contain approximately 500g of organic sunflower (*helianthus*) seeds, two water tubes (carbon filtered water) with cotton wool bung, and a piece of cotton wool substrate.

Stock cages were rotated at random within these incubators (as were experimental trays) to reduce any experimental bias derived from incubator position. In Chapter 3, experimental bugs were housed in (8cm) Petri dishes containing seeds and a 1.5ml Eppendorf tube filled with water and capped with a cotton wool bung. For later experiments, small plastic pots with perforated lids containing seeds and water soaked cotton wool were used, as they required less upkeep and were better at avoiding mould growth due to humidity etc. (see Figure 7, and respective methods for precise details). Large mouth pooters were used to select and transfer bugs, in volume, for culturing stock cage populations. For the experiments, soft, storkbill, forceps were used to manoeuvre bugs, but bugs were otherwise handled as little as possible.

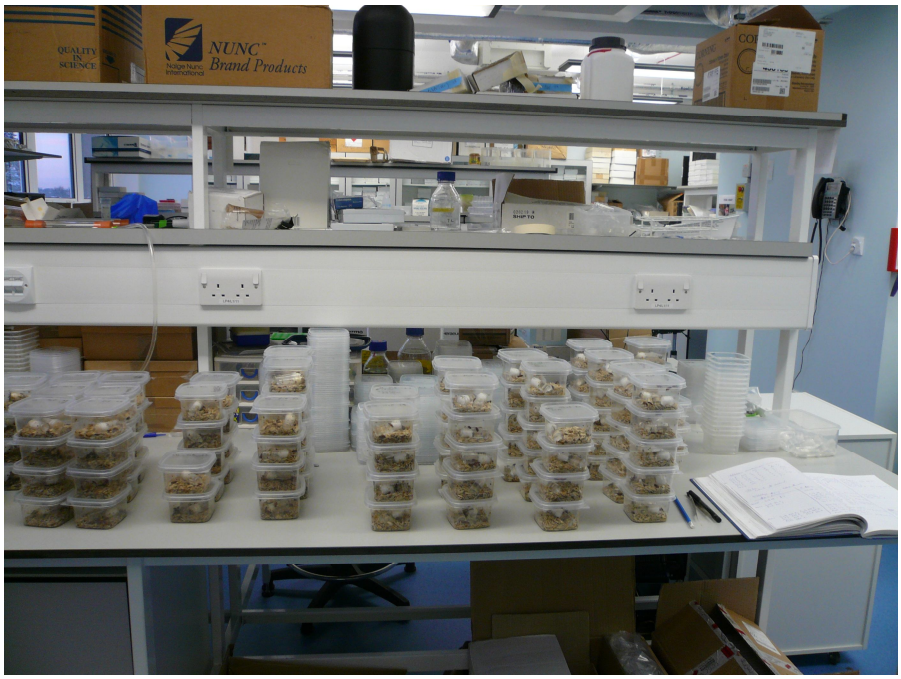


Figure 7. Experimental pots of *Lygaeus* bugs, containing a layer of seeds, and a water tube similar to the large population cages shown in Figure 5.

Chapter 3

Receptivity to mating and reproductive development in the seed bug *Lygaeus equestris*

Abstract

Receptivity to mating has important implications for the evolution of mating systems, particularly in light of recent developments in sexual conflict theory. Where sexual conflict over mating exists, female receptivity may be a mechanism by which females retain control over mating decisions in response to male manipulation. Here I explore patterns of initial (or primary) receptivity to mating across early adult development in two populations of the seed bug *Lygaeus equestris*, known to differ in female mating costs. Male reproductive development (scored by seminal vesicle width), was faster than female reproductive development (measured by oocyte production), but did not differ between the populations. Female development was faster in the "Leeds" population, which exhibits higher mating costs, and bugs from this population (both male and female) mated more readily. Over the age range studied, in both populations a greater proportion of females mated to reproductively mature males than did males to reproductively mature females. Additionally I considered the genetic basis of initial female receptivity to mating for two age groups in the "Dolomite" population. In both cases, the heritability of receptivity was moderate to low and not significant, and receptivity did not differ significantly between the groups.

Introduction

Receptivity to mating has important implications in evolutionary ecology from mating behaviour, conflicts of interest between the sexes, through to mating system evolution itself (Choe and Crespi 1997; Arnqvist and Rowe 2005; Hosken *et al.* 2009). It is a key component in determining the likelihood of mating a random individual of the opposite sex upon an encounter, and will interact with processes of mate choice and consequently affect patterns of sexual selection (Bateson 1983). In many species, males typically initiate mating, as they tend to gain more from additional matings than females (Bateman 1948; Trivers 1972; Arnqvist and Rowe 2005). Females are usually considered to be the choosy sex that males compete for (Bateson 1983; Andersson 1994; Arnqvist and Rowe 2005), and work on receptivity to mating has focused on females (Ringo 1996), with male willingness to mate viewed more in terms of male 'persistence' (Arnqvist and Rowe 2005). Male persistence in attempting to mate then interacts with female receptivity leading to either successful mating, or rejection. Thus, the interaction between male willingness to mate and female receptivity is central to sexual conflict over mating, sexually antagonistic evolution, and consequently mating system evolution.

Conspecific males and females must exchange long-range sexual advertisement, and/or short-range courtship signals to locate partners, and achieve mating. Such signals range from chemical advertisements to prolonged, dance-like, rituals and may encompass many sensory modalities (Ringo 1996; Alexander *et al.* 1997). Acceptance of a mating partner by a female may involve active behavioural cooperation (as in some crickets where females mount males prior to genitalic engagement: Mays 1971; Gwynne 1981) or may simply manifest itself as an absence of rejection behaviour (e.g. seaweed flies, Shuker and Day 2001; see also Ringo 1996). For females, three basic mating patterns are observed in nature. Females may be receptive: (1) for a brief period only; (2) continuously or; (3) cyclically, throughout their lives (Ringo 1996). Generally monandry, where females mate with just one partner (and perhaps only once in their lifetime), will be more common when females are receptive for a short period only, whilst polyandry will be more frequent in the two remaining mating patterns. The majority of insects display cyclical receptivity (Ringo 1996).

In genetic terms, willingness to mate (for both males and females) is likely to be a product of a wide array of intrinsic, physiological and neurological factors, as well as having considerable environmental influences (reviewed by Ringo 1996; Torres-Vila *et al.* 1997). For example, in Lepidoptera, female size and nutrition, as well as male quality (including spermatophore size), and the abundance of mates are known to affect patterns of receptivity (Torres-Vila *et al.* 1997; Torres-Vila *et al.* 2005; Wedell 2005). Certain socio-environments may be required to initiate sexual receptivity in some systems, as is apparent for pair-bond formation in the monogamous prairie vole, *Microtus ochrogaster*. Here, prolonged contact with a novel male is necessary to initiate ovarian activity and female receptivity in order to ensure successful reproduction, all of which is mediated by neuroendocrine mechanisms, including the neuropeptide vasopresin (Roberts *et al.* 1998; Young *et al.* 2001; Lim and Young 2004).

Although female receptivity can develop in association with reproductive development (Ringo 1996), copulation stimulates the reproductive development of females in many species (Arnqvist and Nilsson 2000). Furthermore, mating itself commonly reduces female post-mating receptivity (increasing the latency to re-mate), as can the presence of sperm in the spermatheca, and accessory proteins transferred to females in the ejaculate (e.g. Gromko *et al.* 1984; Andersson *et al.* 2000; Arnqvist and Nilsson 2000; but see Chapman *et al.* 1998 for exceptions). On the other hand, the amount or intensity of male courtship can also influence the probability of mating and re-mating, and so males may invest substantially in courtship displays (Ringo 1996; Hunt *et al.* 2004a; Shamble *et al.* 2009; Crudgington *et al.* 2010). These effects highlight female receptivity as a function not only of female physiology, but also of male attempts to control female physiology (Wolfner 2009).

Here I explore patterns of initial receptivity to mating (mating propensity) for both males and females across early adult reproductive development in two populations of the seed bug, *Lygaeus equestris*, when paired with a sexually mature (assumed from previous observations, and see Figure 7) virgin of the opposite sex. Despite being highly promiscuous, females of this species incur high mating costs, indicative of sexual conflict (Sillén-Tullberg 1981; Solbreck *et al.* 1989; Shuker *et al.* 2006). More specifically, Shuker et al (2006) found that high mating rates resulting from retaining females with three males led to substantial fitness costs to these females, in terms of reduced longevity and fecundity, compared to females mated only once, or retained with one male throughout life. These fitness costs to females were associated somewhat with male harassment but were much

greater when copulation itself was included (Shuker *et al.* 2006). Furthermore, the two populations considered here vary in the magnitude of female mating costs, with females housed with three males surviving approximately 40% and 50% as long as singly mated females, and producing approximately 13% and 38% as many eggs in each population respectively (see Shuker *et al.* 2006). The populations also varied in many other life history traits (Solbreck *et al.* 1989; Shuker *et al.* 2006, this study), making them ideal candidates for studying patterns of in initial receptivity.

Specifically I consider how populations known to vary in female mating costs vary in their male and female reproductive development, and how this relates to initial receptivity to mating. Härdling and Kaitala (2005) demonstrated theoretically that the predicted remating probability of females in a population (remating rates that maximise female fitness and so cannot be invaded by other remating strategies) depends on the marginal costs and benefits of mating, and not on population density itself, and that the remating probability is always lower with greater relative mating costs. Given the co-evolutionary nature of male-female interactions, definitive predictions are hard to make. However, from previous work concerning differences between *Lygaeus equestris* populations in female costs of mating, for the first experiment I predicted that: (1) females from the 'Dolomite' population, that are subject to lower mating costs (Shuker *et al.* 2006) would be more receptive (less resistant) to males than females of the 'Leeds' population; (2) male willingness to mate would not differ between the populations (assuming a similar high optimum mating rate for both populations); (3) both male and female receptivity would vary with reproductive development, given the obvious constraints of maturing primary sexual function in adulthood. I then address the heritability of female initial receptivity in one of the study populations to determine and explore the genetic basis, and evolvabiltiy, of female receptivity at two distinct age classes, early mating and late mating (females aged 6 and 12 days post adult eclosion) respectively. Early mating here represents mating at an early stage of reproductive maturation (where females may gain from mating in terms of enhancing their own reproductive development, but see Kugelberg 1973b) whilst later mating females (at 12 days post adult eclosion) are reproductively mature (see below).

Materials and Methods

Lygaeus equestris biology

The seed bug *Lygaeus equestris* (Hemiptera: Lygaeidae) is a widely distributed, aposematic seed predator occurring across much of Europe (Solbreck *et al.* 1989; Péricart 1998). It feeds on the flower ovules and seeds of many plant species including its preferred host-plant *Vincetoxicum hirundinaria* (Gentianales: Asclepiadaceae)(Solbreck and Kugelberg 1972; Kugelberg 1973b; Kugelberg 1973c; Kugelberg 1974; Solbreck *et al.* 1989). In the laboratory, at 29°C and a 18:6 L:D cycle, the generation time from egg to adult is between 4-5 weeks on shelled *Helianthus* seeds, and adults can live for up to three months under these conditions (Shuker *et al.* 2006).

Lygaeus equestris is a highly promiscuous species, with both sexes mating multiply throughout adult life (Solbreck 1972; Sillén-Tullberg 1981; Shuker *et al.* 2006). The extent of female control of mating is unclear (see Chapter 2 and discussion), and pre-copulatory struggles (between females and males attempting to mate) are often observed, but fertilization failures appear to be common in the closely related *L. simulans* (Tadler *et al.* 1999; Micholitsch *et al.* 2000). Males grasp and mount females dorsally before rotating their abdomen under the female and attempting to engage genitalia in a stable end-to-end position, before inserting the aedeagus into the females' bursa copulatrix (Sillén-Tullberg 1981; see also Tadler 1999; Tadler *et al.* 1999; Micholitsch *et al.* 2000; Huber *et al.* 2007). Mating also functions as a facultative anti-diapause mechanism in *L. equestris* (Sillén-Tullberg 1984), indicating lasting physiological effects associated with mating. See Chapter 2 for further details of *L. equestris* biology.

Experimental setup

I used two populations of *L. equestris* originating from different geographical regions for the experiments described here: one from northern Italy in the Dolomite region (the Dolomite population, sampled in 2004), and another originally collected from Sicily in southern Italy and established at the University of Leeds in 1996 (the Leeds population, Shuker *et al.* 2006). I performed the first experiment from November to December 2007, and the quantitative genetic experiment in from January to April 2008.

Prior to the experiments, late instar nymphs were transferred into large 'nymph cages', containing seeds, water and cotton wool as per the culturing cages (see Chapter 2 for general husbandry), but with no adults. I removed freshly eclosed adults daily from these nymph cages and separated them into single-sex Petri dishes, at a maximum density of eight bugs per dish, ensuring virginity. Bugs were retained in these Petri dishes until required. All Petri dishes contained organic *Helianthus* seeds *ad libitum*, a 1.5ml Eppendorf tube filled with water and capped with a cotton wool bung, and a small piece of cotton wool. As with the stock cages, water tubes were changed regularly. This was repeated for both populations.

Reproductive development

Reproductive development was quantified for both males and females of both populations. Adult males and females were dissected at six different age groups: 2, 4, 6, 8, 10, and 12 days post adult eclosion. Between 6 and 11 bugs per age group were dissected for both sexes and for both populations (median sample size = 10, total $N = 236$). The experimental period spanned six weeks of adult emergence from the nymph cages. Males and females of all post-eclosion ages were isolated and dissected together in blocks.

Male reproductive development ($N = 119$) was scored by a measure of seminal vesicle development on a 1-3 scale. Using a dissecting stereomicroscope fitted with an eyepiece micrometer (x10 magnification), I measured the width of the seminal vesicle immediately below the testes. Bugs were classed as having: (1) no sperm when the seminal vesicle width was ≤0.2mm, and translucent; (2) some sperm; 0.2mm< vesicle width <0.45mm, semi translucent; and (3) lots of sperm; vesicle width ≥0.45mm, white (see Figure. 1). Female reproductive development ($N = 117$) was simply scored as the presence or absence of oocytes in the ovaries (see Figure 2). I recorded the sex, date of eclosion, date the assay was performed and wet mass immediately prior to assaying testes score, or oocyte presence, for males and females respectively. Due to an oversight, the first batch of individuals ($N = 20$) were not weighed prior to dissection.

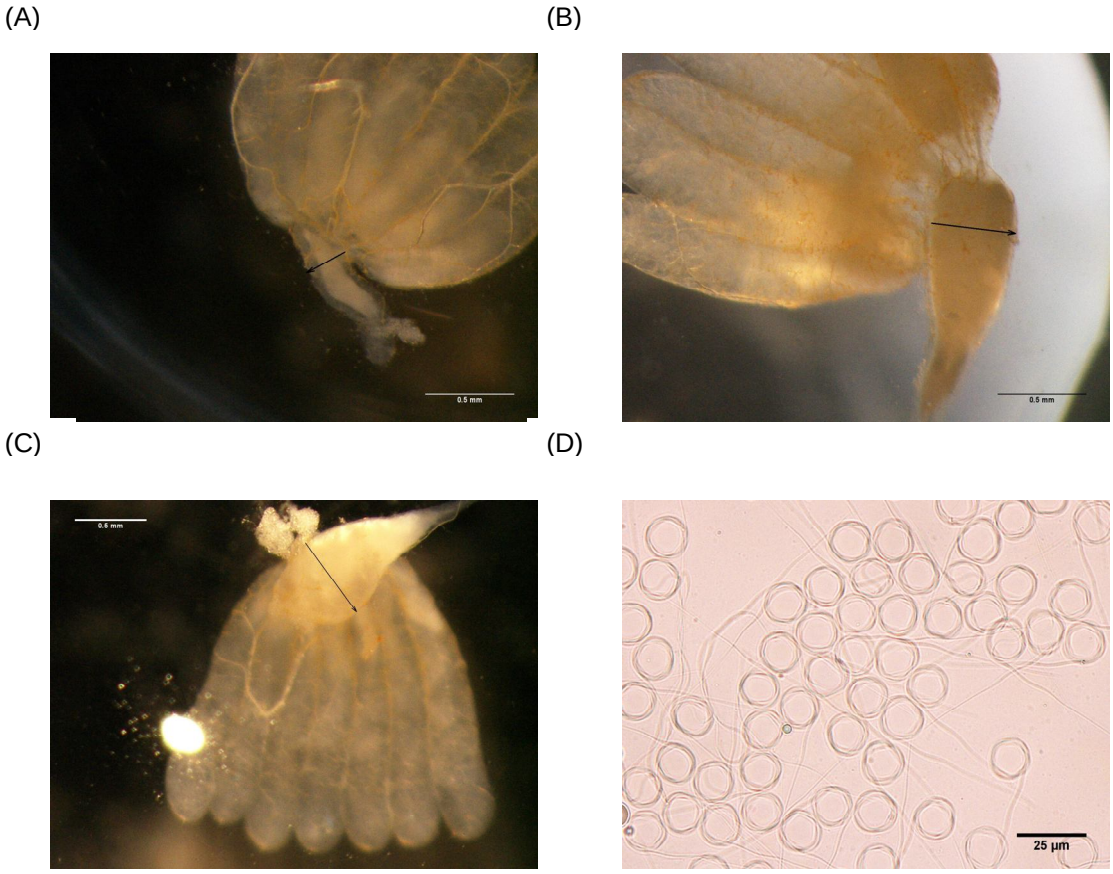


Figure 1. Male reproductive development was scored on a 1-3 scale, depending on the width of the seminal vesicle immediately below the testes (represented by arrows above). (A) testes score 1, vesicle width <0.2mm, (B) testes score 2, vesicle width 0.2- 0.45mm (C) testes score 3, vesicle width >0.45mm. (D) sperm cells within the seminal vesicle.

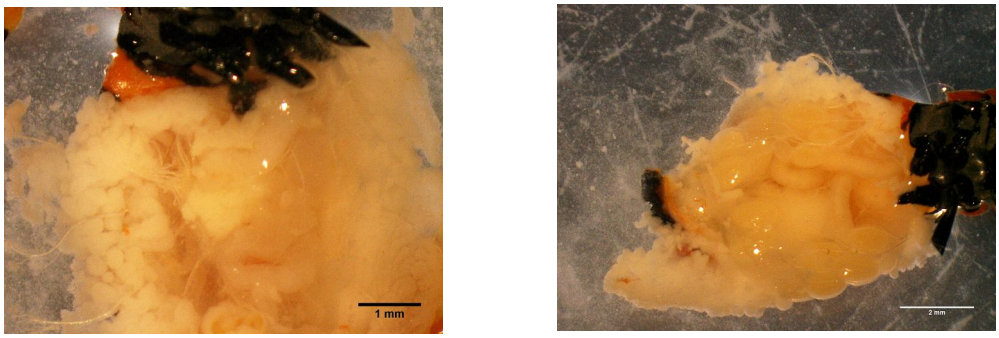


Figure 2. Female reproductive development scored on a binary scale of (A) oocytes absent, and (B) oocytes present.

Receptivity to mating

Across the same age-groups (2 - 12 days post eclosion), I assayed adults of each sex and population for their receptivity to mating. Adult bugs were paired with a virgin non-focal individual of the opposite sex (from the same population), aged between 14 and 22 days post adult eclosion (assumed to be reproductively mature from previous work). These bugs were paired in an empty Petri dish (9cm diameter) for 8 hours and scored for copulation (see Tadler *et al.* 1999) every 30 minutes. From these scan samples I defined three measures of mating activity: (1) 'successful' copulations, where pairs were observed mating (end to end genitalic engagement) for at least three consecutive observations (suggesting a minimum copulation duration of 60 minutes, approximating the minimum duration necessary for successful sperm transfer to occur in the closely related *Lygaeus simulans* (but see Sillén-Tullberg 1981; Tadler *et al.* 1999); (2) 'long' copulations, where pairs were observed mating for at least eleven consecutive scans (approximating 5 hours duration), ample time for successful insemination to have occurred (Sillén-Tullberg 1981; Tadler *et al.* 1999; Shuker *et al.* 2006); and (3) 'sexually active', whether or not any sexual activity (genitalic engagement) was scored at all. Approximately 9 replicate bugs were obtained per age treatment for each sex and population (range = 7-10, total $N = 214$).

Quantitative genetic basis of female mating receptivity

To examine the genetic basis of female primary receptivity I conducted a standard half-sib–full-sib breeding experiment on bugs from the Dolomite population (Figure 3). Sires (aged 7-12 post adult eclosion; $N = 22$), were mated to two to three randomly assigned dams (aged 7-8 days old; $N = 47$). Sires were mated to dams every other day to reduce potential sperm depletion. Sire-dam pairs were placed in empty Petri dishes (9cm diameter) and scored for copulation every hour for seven hours. Females were isolated from males upon separation from mating, to ensure (as far as possible) a single copulation event. Prior to mating, dams were retained in same-age groups in Petri dishes (9cm diameter) with excess food and water. Males were similarly retained until required for mating, whereupon they were kept in isolation before mating further females.

After mating, females were retained individually in Petri dishes with excess food, water and a piece of cotton wool as an oviposition substrate. Dams were transferred to a new

dish, after each of her first two clutches were laid, and left in a 3rd dish to continue oviposition. Approximately 20 nymphs were retained per clutch in an attempt to gain 6-8 adult females from each clutch.

Clutches were checked daily for adult eclosion. Newly emerged females were placed in new dishes with food and water in order to keep a record of their (post adult eclosion) age. Adult females originating from the same clutch were retained together. The receptivity to mating of females from this offspring generation was measured at 2 distinct age groups; at 6 days ($mated_6$), or 12 days ($mated_{12}$), post adult eclosion respectively. This covered the age range studied in the previous experiment, from where females first begin to produce oocytes, see Figure 6). Ultimately 2 to 5 female offspring per clutch were placed in each treatment group (both treatments; median = 2, total N = 462). Randomly assigned virgin, non-focal, male partners were aged between 12 and 15 days old. Pairs were scored for mating as described for the parental generation.

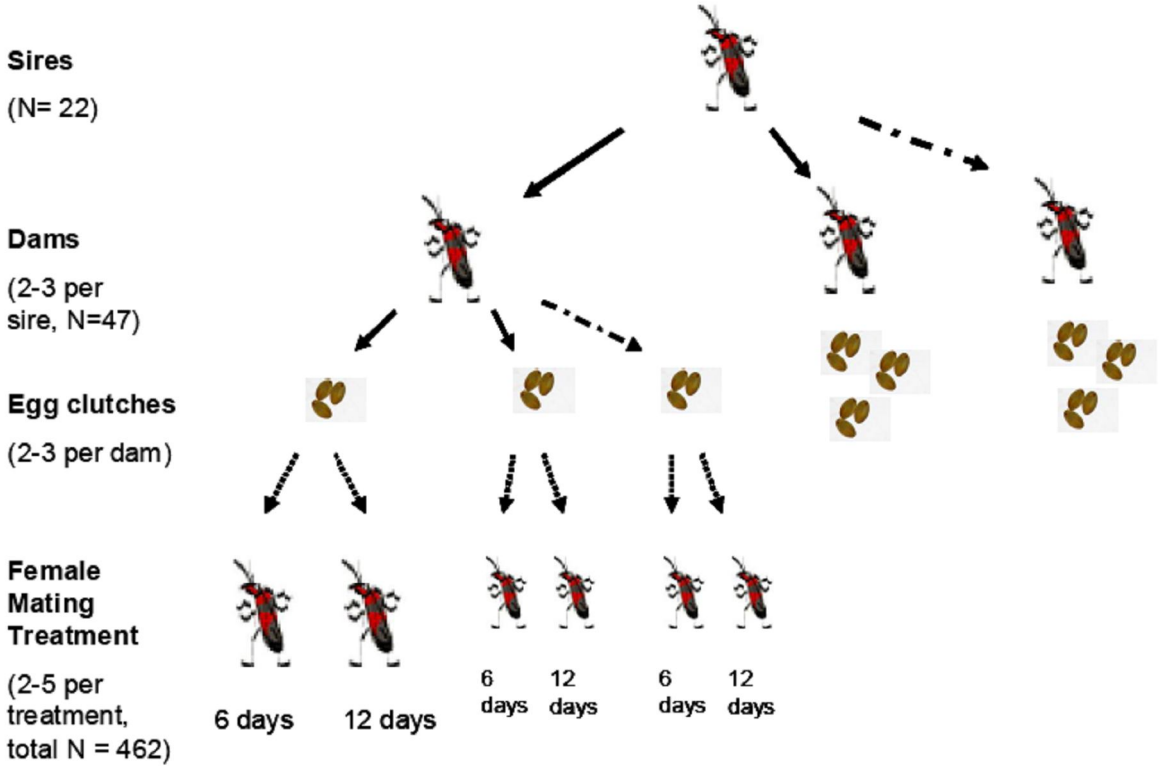


Figure 3. Illustration of the full-sib-half-sib breeding design used for the quantitative genetic experiment of female primary receptivity to mating at 6 and 12 days post adult eclosion respectively. 21 sires were mated to 2 to 3 dams each, from whom 2-3 egg clutches were collected, whereupon adult females were randomly split into mating trials at 6 and 12 days respectively. See text for further details.

Statistical analysis

I used a general linear modelling (GLM) approach for the analysis. Given recent misgivings about step-wise approaches to model simplification (e.g. Whittingham *et al.* 2006; Mundry and Nunn 2009), I fitted full models with the relevant terms for each model and used Type III sums of squares of these models for significance testing. When the latter was not easily applicable, in particular for generalised linear models, I fitted full models and obtained significance tests for terms in the model when each term was fitted last at each level of the model hierarchy (main effects, first order interactions, second order interactions etc). When appropriate, I created sub-models (for instance within each sex or population) to explore particular patterns of interaction between variables. To avoid over-parameterisation of full models (and so-called "fishing trips", Mundry and Nunn 2009), derived variables such as quadratic terms were only included when involved in one or more significant terms in full models (either alone or as part of interaction terms). In short, I tried to be pragmatic with what terms were entered into models.

For the analysis of male reproductive development, I used a generalised linear model (GLIM) with a quasi-poisson error structure and a log link function, and *F* tests for significance testing, as the data were under-dispersed for a standard GLIM with Poisson errors (Crawley 2007). For the analysis of female reproductive development and the three measures of receptivity, I used logistic regression for these presence/absence data. Logistic regression is a GLIM with a binomial error structure and logit-link function, and I tested significance either with likelihood ratio χ^2 tests or, *F* tests if the data were over-dispersed (Crawley 2007). For the analysis of mass over early adult life with respect to sex and population, data were pooled from the reproductive development and receptivity experiments. All statistics were performed with S-Plus 7.0 (Insightful Corporation, Seattle, USA).

Quantitative genetic analysis

A total of 462 females from 22 sire, and 47 dam, families were used to test the quantitative genetic basis of primary (virgin) female receptivity to mating in a half-sib – full-sib experimental design (Figure 3). Female mating propensity, being binary, was modelled throughout as a threshold character, assuming a continuous underlying distribution, or

liability (Roff 1997). Heritability and standard errors were estimated on the observed (0,1) scale before converting to an estimate on the underlying scale (Falconer and Mackay 1996; Roff 1997; Lynch and Walsh 1998). Animal models (linear mixed effect models attached to a pedigree), were fitted to estimate variance components and their corresponding standard errors using restricted maximum likelihood with the program AS_{REML} v 2.0 (Gilmoure *et al.* 2006). An animal model approach is useful for unbalanced designs as it optimizes information use when estimating quantitative genetic parameters (Kruuk 2004; Wilson *et al.* 2009). Univariate mixed effect models were fitted firstly incorporating all the data (i.e. data from both the 6 day and 12 day mating treatments combined) with female age as a fixed effect. Secondly, univariate models were fitted to the two respective mating treatments in isolation. Finally a bivariate model (2-trait model including $mated_6$ and $mated_{12}$ treatments respectively) was then fitted to estimate the genetic correlation between the mating treatments (Wilson *et al.* 2009). Log-likelihood ratio tests were used to calculate the significance of additive genetic effects. I extended the models to include clutch and dam parameters independently as random effects. As with additive genetic effects, maternal (dam) and environmental (clutch) effects were tested by comparing models with and without the fixed effects.

Heritabilities were calculated directly from the animal models, such that $h^2 = V_A / V_P$ (where h^2 is the narrow sense heritability, V_A is the additive genetic variance and V_P is the sum of the variance components), and were subsequently converted onto the underlying scale:

$$h^2{}_u = h^2{}_{obs}\left[p(1-p)\right] / z^2$$

Where $h^2{}_u$ is the heritability on the underlying scale, $h^2{}_{obs}$, is the heritability estimated on the observed (0,1) scale, p is the mean incidence, and z is the corresponding ordinate of p on the standardized normal curve of the underlying scale, and is calculated as:

$$z = \left[e^{\left(-0.5X^2\right)}\right] / \sqrt{2\pi}$$

where, $X = \left[sign(0.5-p)\right]\left[1.238c(1+0.0262c)\right]$ and $c = \sqrt{-\ln\left[4p(1-p)\right]}$

The approximate standard error of the heritability estimate on the underlying scale is similarly obtained:

$$s.e.\left(h^2{}_u\right) = s.e.\left(h^2{}_{obs}\right)\left[p(1-p)\right] / z^2$$

where $s.e.(h^2_u)$ is the approximate standard error of the underlying heritability estimate, $s.e.(h^2_{obs})$ is the standard error corresponding to the heritability calculated on the observed (0,1) scale and z is calculated as above.

Results

Descriptive statistics

Across the two experiments, *L. equestris* wet mass ranged from 0.025g to 0.057g (N = 411, mean = 0.039g, se = 0.0003). Bugs from the Dolomite population were significantly heavier than those from the Leeds population (GLM: $F_{1,403} = 7.94$, $P = 0.005$; Table I) and across the two populations females were also significantly heavier than males ($F_{1,403} = 20.50$, $P < 0.0001$). Female mass increased with age ($F_{1,203} = 7.69$, $P = 0.006$; Figure 4) and this relationship did not vary between the two populations (interaction between population and age: $F_{1,203} = 0.001$, $P = 0.97$). All told, females from the two populations did not significantly differ in mass ($F_{1,203} = 2.97$, $P = 0.09$). Male mass, however, showed no significant relationship with age ($F_{1,200} = 3.02$, $P = 0.08$; interaction between sex and age for the two populations: $F_{1,403} = 10.47$, $P = 0.001$; Figure 4). Dolomite males were slightly heavier than Leeds males ($F_{1,200} = 5.33$, $P = 0.02$; Table I) and this did not vary with age ($F_{1,200} = 1.19$, $P = 0.28$). The difference between males and females in how mass changed with age did not differ between the populations (i.e. no significant three-way interaction: $F_{1,403} = 0.55$, $P = 0.46$).

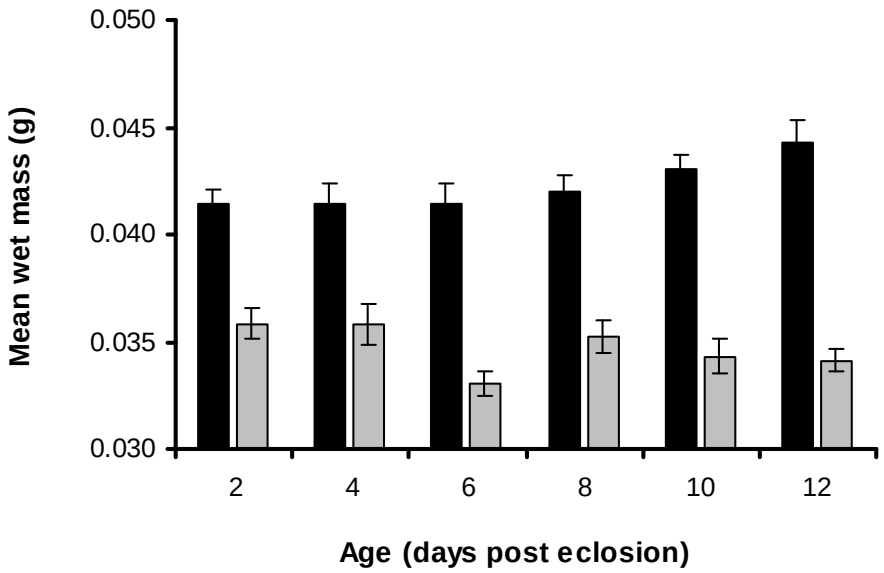


Figure 4. Mean wet mass (g) of males (grey) and females (black) against age for bugs from both the Leeds and Dolomites populations. Error bars are standard errors.

Table I. Mean wet mass (g) ± se and sample size (*N*) for both male and female bugs from the Dolomites and Leeds populations respectively, for the sexes combined within each population, and for the both sexes overall (populations combined).

Population	Sex	*N*	Wet mass (mean, g)	*se*
Dolomites	male	104	0.036	0.0004
	female	105	0.044	0.0004
	combined	209	0.040	0.0004
Leeds	male	100	0.034	0.0004
	female	102	0.041	0.0005
	combined	202	0.037	0.0004
Combined	male	204	0.035	0.0003
	female	207	0.042	0.0004

Reproductive development

Males

Sperm was observed from 2 days post-adult eclosion (i.e. testes score = 2). Most males displayed some sperm by day 6, and almost all males displayed a large volume of sperm by day 12 (testes score = 3; Figure 5). As such, male reproductive development was strongly positively associated with age across the twelve days ($F_{1,116}$ = 319.6, P < 0.0001; Figure 5) and did not differ between the two populations ($F_{1,116}$ = 2.20, P = 0.14). There was also no difference in the association between testes score and age for the two populations (interaction term: $F_{1,115}$ = 1.29, P = 0.26).

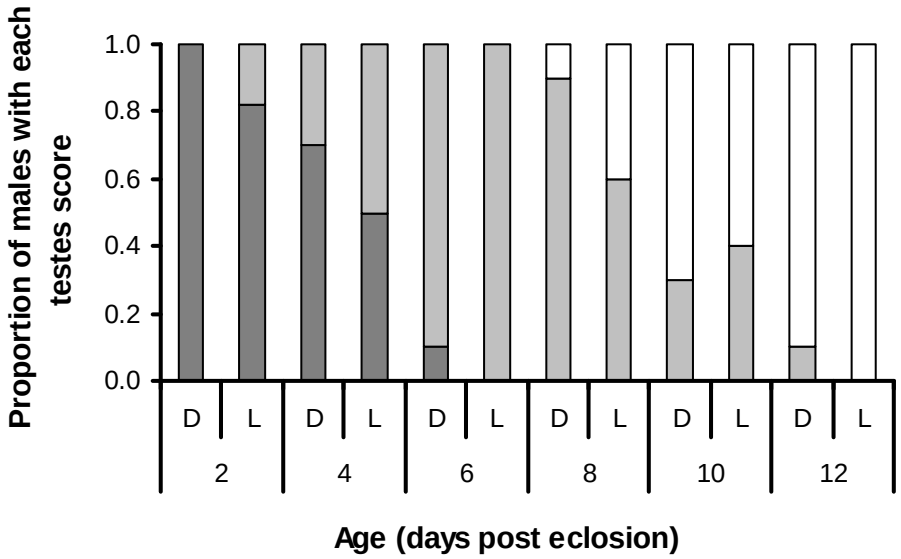


Figure 5. Male reproductive development for the Dolomites (D) and Leeds (L) populations respectively. Proportion of bugs sampled at each age class with testes score 1 (no sperm: dark grey columns), 2 (some sperm: light grey columns), and 3 (lots of sperm: white columns) as measured by the width of the sperm duct immediately below the testis (see text for details).

Females

Reproductive development (as measured here) was slower for females than males. Females displayed oocytes from day 6 onwards, with none present before this (Figure 6). Many females did not display oocytes as late as 12 days old. Oocyte presence increased with age, following a significant quadratic relationship (linear term: $\chi^2_1 = 34.15$, $P < 0.0001$; quadratic term: $\chi^2_1 = 8.48$, $P = 0.004$, Figure 6). Over the whole experiment significantly more Leeds females contained oocytes than Dolomite females (proportion of females with oocytes: Dolomites = 0.20, Leeds = 0.36; $\chi^2_1 = 7.25$, $P = 0.007$). Despite egg development appearing to follow different trajectories for the two populations (Figure 6), there was no significant difference between the populations in the accrual of eggs with age (interaction with the linear term: $\chi^2_1 = 0.78$, $P = 0.38$; interaction with quadratic term: $\chi^2_1 = 1.08$, $P = 0.30$).

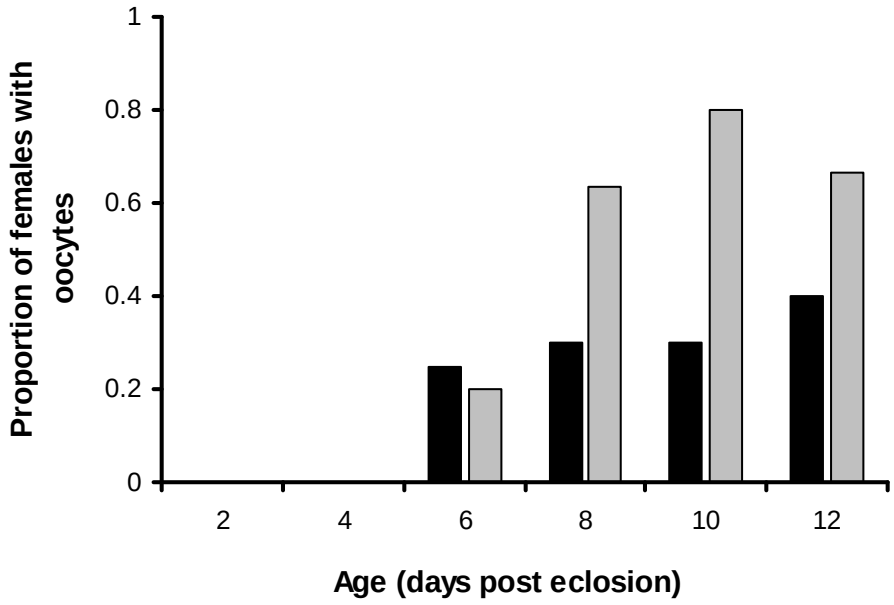


Figure 6. Female sexual development for the Dolomites (black) and Leeds (grey) populations respectively, as measured by the proportion of females at each age group containing oocytes.

Receptivity to mating

Overall, the incidence of successful copulations (3 or more consecutive observations *in copula*) was greater in the Leeds population (proportion mated: Leeds = 0.46; Dolomites = 0.33, logistic regression: $F_{1,212} = 4.37$, $P = 0.038$, Figure 7). In addition, focal females (i.e. females manipulated for age and mated to "mature" males) mated more frequently than focal males (mated to "mature" females; proportion mated: focal males = 0.24, focal females = 0.55; $F_{1,212} = 21.83$, $P < 0.0001$), which was true for both populations (interaction term: $F_{1,211} = 1.03$, $P = 0.31$).

Age post-adult eclosion also influenced the sex- and population-specific patterns of receptivity for focal individuals. Mating occurred earlier for females than for males, with females mating non-focal (12+ day old) males from 2 days post-eclosion, whilst males mated non-focal females from day 6 (Figure 7). As such, both age post-eclosion and the interaction between age and sex were highly significant in the model (age: $F_{1,211} = 6.59$, $P = 0.01$; age*sex interaction: $F_{1,208} = 21.93$, $P < 0.0001$). As above, both sex and population were

significant as main effects (sex: $F_{1,211}$ = 24.32, P < 0.0001; population: $F_{1,211}$ = 4.93, P = 0.03). However, the incidence of successful copulation with respect to age did not differ between the populations (age*population: $F_{1,208}$ = 0.0004, P = 0.98; age*population*sex: $F_{1,207}$ = 1.93, P = 0.17). Thus, across the developmental period tested, females were more likely to mate than males when paired with mature partners, and male and female mating propensities followed different trajectories across the range of ages assayed.

In terms of the occurrence of prolonged copulations (pairs *in copula* for more than 5 hours), again focal females were more likely to be involved than focal males (proportion mated: focal males = 0.29; focal females = 0.61: χ^2_1 = 15.83, P < 0.0001), and again Leeds individuals – both male and female - were more likely to be involved than Dolomites individuals (proportion mated: Leeds = 0.54 ; Dolomites = 0.37, χ^2_1 = 6.61, P = 0.01; sex*population interaction: χ^2_1 = 0.34, P = 0.34, Figure 7C). However, there was no direct effect of age (χ^2_1 = 1.42, P = 0.23; Figure 7C), although the difference between the sexes did change with age (χ^2_1 = 17.22, P < 0.0001).

Finally, in terms of whether individuals were sexually active at all or not, a broadly similar pattern emerges. The two populations and the two sexes differed as before (Leeds > Dolomites: χ^2_1 = 15.04, P = 0.0001 and Females > Males: χ^2_1 = 27.40, P < 0.0001). In addition, the incidence of sexual activity increases with days post-eclosion (χ^2_1 = 14.00, P = 0.0002; Figure 7A), with focal females increasing the likelihood of activity more rapidly than focal males (age*sex interaction: χ^2_1 = 23.89, P < 0.0001).

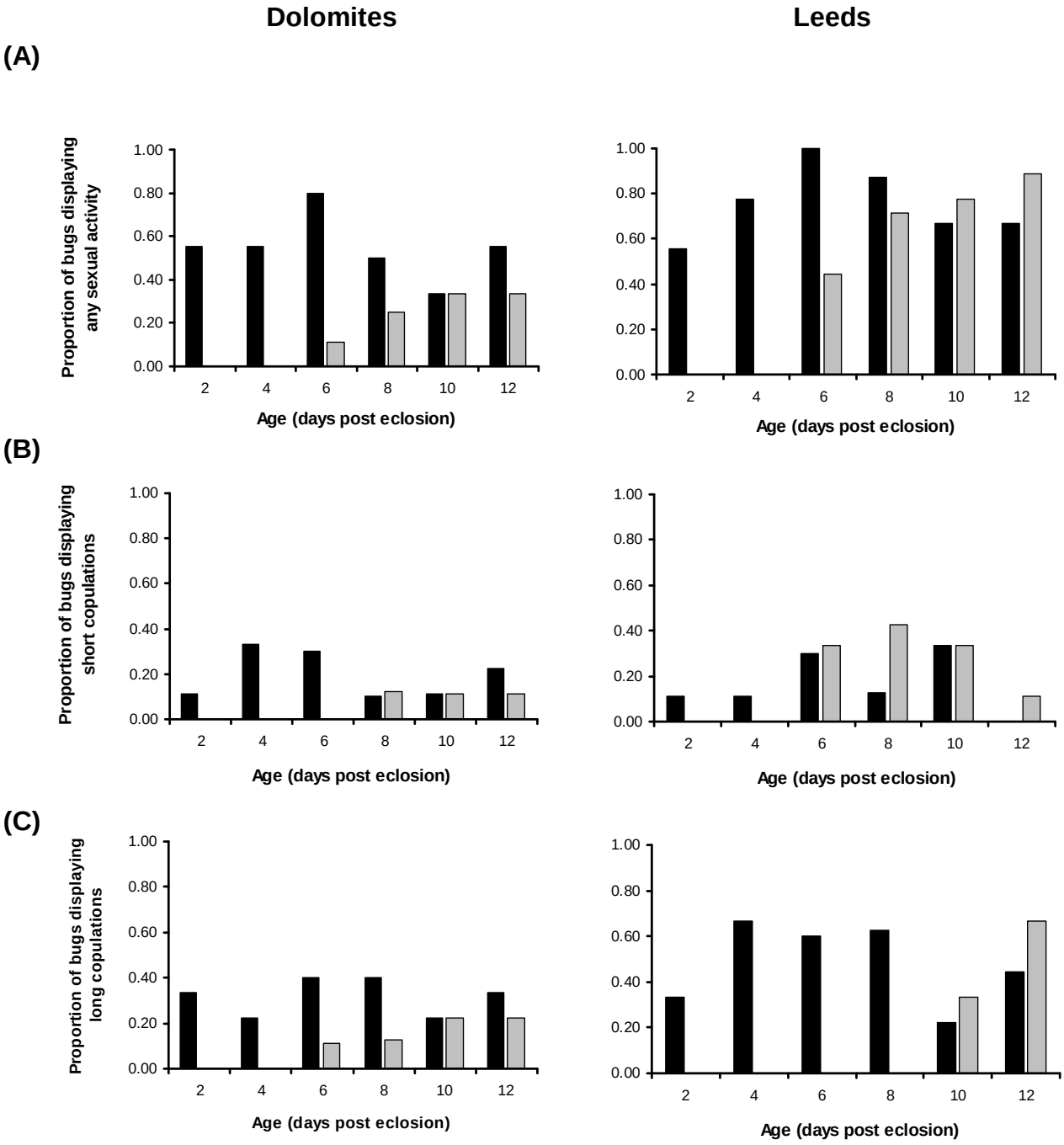


Figure 7. Proportion of bugs displaying the three measures of sexual receptivity: (A) any sexual activity; (B) short copulations; (C) long copulations, for each age group. See text for the behavioural details. Populations are split into pairs of graphs: Dolomite population to the left and the Leeds population to the right. Black bars are females and grey bars are males.

Genetic basis of female receptivity

Mating propensities were high for both traits considered here (mean incidence: $mated_6$ = 0.89; $mated_{12}$ = 0.83; total incidence 0.86). There was no significant difference between mating propensity at the two age treatments ($F_{1,460}$ = 2.99, P = 0.085). Heritability was not high for $mated_6$ (h^2_{obs} = 0.093, se = 0.099; h^2_u = 0.257, se = 0.279), and this was associated with a high standard error, as was the case for all heritability estimates (Table II). Large residual error was apparent in all models and no significant additive genetic effects were found (Table II). The bivariate analysis revealed a non-significant, negative, genetic correlation between the two mating treatments ($mated_6$ and $mated_{12}$) respectively (COV_A= -0.0014, se = 0.0070; r_G = -0.3545, se = 1.9626), and yielded similar additive genetic effect estimates to the univariate analyses for the two traits modeled (Table III). In all cases, there were no maternal or clutch effects (log-likelihoods of models with dam and clutch added to models independently yielded no change in the model likelihoods, data not shown).

Table II. Quantitative genetic analysis of female mating propensity in total (mated) and at two age classes – 6 ($mated_6$), and 12 ($mated_{12}$), days post adult eclosion respectively using animal models in ASReml. Variance components and their standard errors are shown for univariate models (including age as a fixed effect for the model using the whole data set). Heritabilities and their approximate standard errors are shown on the observed (h^2_{obs}) and underlying (h^2_u) scales.

	V_P	se	V_A	se	V_R	se	h^2_{obs}	se	h^2_u	se
Mated	0.119	0.008	0.002	0.005	0.117	0.009	0.014	0.039	0.035	0.097
$Mated_6$	0.099	0.009	0.009	0.010	0.090	0.012	0.093	0.099	0.257	0.279
$Mated_{12}$	0.140	0.013	0.002	0.011	0.138	0.017	0.011	0.080	0.030	0.220

Table III. Variance-co-variance matrix for early ($mated_6$) and late ($mated_{12}$) mating traits respectively. The top left and bottom right values represent the additive genetic variances, V_A for $mated_6$ and $mated_{12}$ respectively. The bottom left value is the estimated genetic covariance between the traits. The top right value is the genetic correlation between the two traits. Numbers in brackets are the standard errors for each term.

	$mated_6$	**$mated_{12}$**
$mated_6$	0.009387 (0.00998)	-0.3545 (1.96260)
$mated_{12}$	-0.001436 (0.00703)	0.00175 (0.01117)

Discussion

Receptivity to mating is important in terms of sexual conflict over mating rate and is a central component of mating system evolution. The aim of this study was to examine how patterns of male and female reproductive development, and initial receptivity to mating, varied between two populations of the seed bug *L. equestris*, and to examine the genetic basis underlying female receptivity in one of these populations. These populations are known to differ in female mating costs, with females of the Leeds population exhibiting greater relative reduction in fitness under elevated mating regimes, suggesting differences in the level, or intensity, of sexual conflict (Shuker *et al.* 2006). Specifically, I asked if initial receptivity to mating is lower for females from a population exhibiting high sexual conflict. Contrary to predictions however, receptivity to sexual activity was higher in the Leeds population (which shows higher mating costs: Shuker *et al.* 2006) for all three receptivity measures (i.e. short and long copulations and any mating activity) over the developmental period studied. An important caveat to acknowledge straight-away is that I have only considered two populations here, and predictions associated with coevolutionary dynamics expected under scenarios such as sexual conflict can be complex (e.g. Holland and Rice 1998; Rice 2000; Gavrilets and Waxman 2002; Gavrilets and Hayashi 2005; Lessells 2006). Nonetheless, I have shown that populations can differ in important mating traits, setting the scene for future work to address patterns of receptivity and costs of mating across a broader range of populations (work that is currently ongoing).

As well as being lighter and smaller than females, males from both populations had faster reproductive development (measured by the proxy of sperm duct width) than females (measured by oocyte presence). Male reproductive development did not differ between the populations, despite Dolomite males being heavier than Leeds males. This suggests that despite the apparent population difference in female mating costs (Shuker *et al.* 2006), male reproductive development does not differ substantially. The rise in focal male mating activity (proportion mating at each age class treatment, Figure 7) corresponds with male reproductive development in terms of increased sperm production. Most males displayed some sperm by day 6, whereupon males of both populations were first observed to engage in mating activity, and the incidence of mating increased in frequency with age thereafter (Figure 7A). Although there was no population difference in male reproductive development, Leeds males mated more readily than Dolomite males. Of course, this is also suggestive of higher

receptivity to mating (lower resistance to males) of mature Leeds females relative to mature Dolomite females, and highlights the difficulties of disentangling these phenotypes.

Conversely, the reproductive development of females did differ significantly between the populations, with Leeds females displaying faster reproductive development as virgins (greater incidence of oocyte presence). Indeed, over the age range tested, Leeds females, that incur higher mating costs (Shuker *et al.* 2006), were more likely to have oocytes present than Dolomite females. Nevertheless, no oocytes were observed in females of either population until 6 days post adult eclosion (Figure 6). In contrast with males, the female mating patterns of both populations did not correspond with female reproductive development, with females mating non-focal (mature virgin males) from 2 days post eclosion. This suggests that female reproductive development (as I have measured it) is not necessary for females to mate, whilst the measure of male reproductive development reflects more faithfully their ability to mate.

An important factor to remember is whether males or females are in control of mating. Given the confounding effect of the sex of the non-focal individual, it is not possible to determine which individual (if any) is in control of the outcome of the mating interactions considered here. However, across the age range tested, the outcomes of the mating interactions do vary depending on the sexes of the focal and non-focal individuals (with females more likely to mate in the former role than males). The age of the focal individual also interacts with this sex difference, but the two populations do not differ in this respect. This sex difference could be associated with a number of factors. Firstly, mature (non-focal) males could be generally more willing to mate than mature females, and may be better able to coerce recently-emerged females to mate. Female *L. equestris* clearly do need to mate as well, however recently eclosed males may make less attractive partners for them. Secondly, males did not mate until 6 days post-eclosion, and even though sperm development is underway, male mating ability is also likely to be limited by other factors. For example, the aedeagus must become fully sclerotized before a male can mate, and necessary accessory products for the ejaculate must also be produced (*L. simulans*, Tadler 1999; Micholitsch *et al.* 2000; *Togo hemipterus*, Himuro and Fujisaki 2008; *L. equestris*, Higgins *et al.* 2009). Similar male and female mating propensities are not observed until approximately 10 days post-eclosion, suggesting males need to attain a certain level of development before (1) being sufficiently attractive to the female to ensure she solicits mating or (2) being able to modify her receptivity and coerce mating (overcome female resistance).

Given the costs associated with mating for females (Sillén-Tullberg 1981; Shuker *et al.* 2006), I predicted that females from the Leeds population have responded to high mating costs by evolving a lower initial receptivity, thus either limiting the likelihood of incurring these high costs through a reduced initial receptivity in the first instance, or by evolving lower receptivity overall. However, of the two populations, Leeds females displayed the higher incidence of mating, countering my prediction. Thus, Leeds females (previously shown to have higher mating costs) displayed faster reproductive development in terms of presence of oocytes, and in terms of copulating with males (sexually mature virgins) more readily. These results may reflect laboratory adaptation of the Leeds population; having been selecting for early reproduction, for example. However, the Dolomites population has also been retained in the laboratory, and cultured in the same manner as the Leeds population, since 2004. Any selection associated with laboratory husbandry should thus be equivalent among the populations, albeit that Leeds population has been held for longer. The Leeds population was originally derived from Sicily, where the potential for multi-voltinism may be higher (Solbreck *et al.* 1989; Shuker *et al.* 2006). Thus, alternatively these results may reflect an adaptation for early reproduction associated with bi-voltinism in the Leeds population, which may not be the case for the Dolomites population with a lower potential for bi-voltinism (Solbreck *et al.* 1989; Shuker *et al.* 2006). However this also raises an interesting and important question as to whether initial receptivity correlates with, and can be informative of, subsequent receptivity (i.e. the level of polyandry). Early studies on *Drosophila melanogaster* indicated that higher receptivity was associated with fast re-mating in lines selected for initial receptivity and re-mating speed respectively (reviewed by Ringo 1996). Furthermore, many species display substantial variation in initial receptivity and also remating speed (Ringo 1996), suggesting that initial, or baseline, receptivity levels could be used as a proxy for measuring the intensity of sexual conflict over mating. We do not yet know if that is the case in *L. equestris*, and this represents an important next step in for future work.

The genetic basis of female receptivity to mating has been explored in a range of insects from fruit flies (Pyle and Gromko 1981; McRobert *et al.* 1995; Sgro *et al.* 1998), parasitoid wasps (Burton-Chellew *et al.* 2007; Shuker *et al.* 2007), bees (Kraus *et al.* 2005), moths (Torres-Vila *et al.* 2001; Torres-Vila *et al.* 2002), butterflies (Wedell 2001; Wedell *et al.* 2002b), crickets (Solymar and Cade 1990; Simmons 2003), to beetles (Harano and Miyatake 2005; see also Evans and Simmons 2008; House *et al.* 2008). Early studies on

moths suggested the presence of large heritable variation for re-mating receptivity (i.e. polyandry: Torres-Vila *et al.* 2001; Torres-Vila *et al.* 2002). However, large dominance variance in mating rates has also been found (Torres-Vila *et al.* 2002; Harano and Miyatake 2005). Indeed, studies that specifically allow for the partitioning of genetic variance between sires and dams suggest low levels of additive genetic variance compared with high dam effects including x-linkage and/or maternal effects (Simmons 2003; Shuker *et al.* 2007; reviewed by Evans and Simmons 2008). Nevertheless sufficient additive variance may still be available for female mating rates to evolve in many species.

I found the heritability of female receptivity in *L. equestris* to be moderate for early ($mated_6$), and low for late mating ($mated_{12}$) treatments respectively, although large standard errors rendered none of the additive genetic effects significant. A moderate negative, but non-significant, genetic correlation was estimated between early and late mating receptivity respectively but again this estimate was associated with large standard error (Table III), and should be interpreted with caution (particularly since the additive genetic effects estimated on the two traits are themselves non-significant). I also found no evidence for maternal or environmental effects. Accordingly, from this admittedly small study of the genetic basis of female receptivity, I was unable to determine the extent of the evolvability of female initial receptivity, and so its potential as a mechanism influencing sexual conflict over mating remains unclear. Thus, although a number of behavioural and life history differences between *L. equestris* populations have now been identified (Solbreck *et al.* 1989; Shuker *et al.* 2006; this study), larger-scale experiments will be needed to resolve within-population genetic architecture of reproductive behaviours.

Receptivity to mating for males and females remain important factors for sexual conflict over mating (Arnqvist and Rowe 2005). I have shown that a population with higher female mating costs also displays faster reproductive development of females and that these females also mate more readily during early adulthood. This counters some expectations from sexually antagonistic co-evolution where we might predict a population under higher sexual conflict (greater female mating costs) to show greater resistance, or reduced receptivity, to male mating attempts. However, this current study is limited to only two populations. In addition, only initial receptivity is considered here and we do not know if this correlates with remating receptivity in this species (i.e. if the female receptivity to mating function is fixed in shape or plastic, but see Ringo 1996), and this warrants further study. I find evidence of only limited heritability of initial receptivity to mating for females, and so

the potential for (co)evolution of female receptivity seems likewise limited, if male-imposed costs of mating do indeed select on female mating behaviour (Shuker *et al.* 2006). However, larger-scale experiments are needed to resolve within-population genetic architecture of female reproductive behaviour and its implications for male-female coevolution.

Chapter 4

No variation in conflict over mating within and between two closely related species of *Lygaeus* seed bugs

Abstract

Sexual conflict and sexual antagonistic co-evolution (SAC) have been proposed as important drivers of evolutionary change and population divergence in the wild. Although the potential for sexual conflict is expected to be ubiquitous in sexually reproducing species, the expression of subsequent SAC in the field may not be inevitable, as selection from other ecological and evolutionary forces may preclude, or swamp, it. Thus, its general importance as an evolutionary force has been challenged. Here I use multiple populations of the seed feeding bugs *Lygaeus equestris* and *Lygaeus simulans* to examine whether field populations differ in the extent of sexual conflict over mating when female mating costs are examined in common garden experiments. I find large costs to females from elevated mating rates using a suite of fitness proxies (symptomatic of sexual conflict). Substantial variation in life history traits, both within and between species, were also observed, and their relation to sexual conflict is discussed. I finish by considering to what extent identifying sexually antagonistic selection among populations moves us towards predicting population divergence as a result of sexual conflict.

Introduction

Sexual conflict can be defined as a conflict between the evolutionary interests of individuals of the two sexes (Parker 1979), and arises whenever male and female fitness optima differ (Parker 1979; Arnqvist and Rowe 2005; Parker 2006). As such, conflict over mating is expected to be ubiquitous among promiscuous species (Parker 1979; Arnqvist and Rowe 2005). Male reproductive success is largely limited by the number of females inseminated, whilst female reproductive success is limited by the number of offspring they can produce (Bateman 1948; Trivers 1972). Moreover, in insects one, or a few, mating events are often sufficient to fertilise the full complement of a female's eggs, with further mating being superfluous and costly for females (Arnqvist and Nilsson 2000; Simmons 2005). This creates conflict between the sexes over mating rate with males expected to be selected to mate more often than is optimal for females (Arnqvist and Rowe 2005).

The potential to drive rapid and divergent evolutionary change via sexually antagonistic co-evolution (SAC) is an important implication of sexual conflict (SAC, Parker 1979; Rice 2000; Arnqvist and Rowe 2005; Lessells 2006). Reciprocal adaptation and counter-adaptation between the sexes, symptomatic of SAC, can result in complex co-evolutionary dynamics (e.g. arms races, Parker 1979; Holland and Rice 1998; Lessells 2006). Numerous factors are likely to be involved in determining interaction outcomes. Thus, sexual antagonism may act simultaneously over multiple traits, involving many loci (Parker and Partridge 1998; Gavrilets 2000; Arnqvist and Rowe 2005). This suggests that there is great potential for sexual conflict to drive population divergence, as distinct populations may evolve along alternative co-evolutionary trajectories, promoting reproductive divergence across populations (Parker and Partridge 1998; Gavrilets 2000; Martin and Hosken 2003; Arnqvist and Rowe 2005). However, sexual conflict may also hinder population diversification, if conflict over mating selects for indiscriminately manipulative males, that then maintain gene-flow among parapatric populations (Parker 1979; Parker and Partridge 1998; Gavrilets and Hayashi 2005).

At one level, the existence of sexual conflict over mating seems to be a feature of sexually reproducing species, particularly with the observation of male harassment of females, male grasping structures, harmful seminal fluid, traumatic insemination, and infanticide (reviewed by Arnqvist and Rowe 2005; Chapman 2006; Lessells 2006). Indeed,

sexual conflict has been invoked to explain much of the variation in reproductive traits observed in nature, from courtship and mating to fertilisation and parental investment (Chapman *et al.* 2003; Arnqvist and Rowe 2005; Chapman 2006). Comparative studies provide compelling evidence for SAC operating in the wild, such as with the correlated evolution of male grasping and female anti-grasping structures respectively (relating to mating rate), independent of phylogenetic relatedness, among species of water striders (Arnqvist and Rowe 2002a; Arnqvist and Rowe 2002b), diving beetles (Bergsten *et al.* 2001; Bergsten and Miller 2007), and plant bugs (Tatarnic and Cassis 2010, see also Koene and Schulenburg 2005; Anthes *et al.* 2008). However, laboratory evolution studies demonstrate most clearly the existence of sexual conflict over mating and the operation of rapid SAC (Holland and Rice 1999; Martin and Hosken 2003; Wigby and Chapman 2004; Stewart *et al.* 2005; Rice *et al.* 2006). For example enforcing monogamy in *Drosophila melanogaster* resulted in reduced conflict between the sexes, as predicted by theory, and greater net fitness (reproductive rate) overall (Holland and Rice 1999). Additionally, artificial selection experiments that acted to increase female resistance to mating showed that increased resistance to mating repeatedly spreads through polygamous populations, with the costs alleviated by the increased resistance outweighing the potential benefits of multiple mating (Stewart *et al.* 2005; Rice *et al.* 2006). Detailed biochemical analysis of mated females, and the action of male derived molecules on female physiology in *Drosophila melanogaster* shows the numerous biochemical pathways with which males may gain by manipulating female physiology, and the effect that sexual conflict and SAC appears to have had on these interactions (Wolfner 2009).

Female mating costs are integral to, and a requirement of, sexual conflict over mating (Lessells 2006). Female mating costs can result from harmful effects of toxic substances transferred in the male's ejaculate (e.g. Chapman *et al.* 1995), the increased risk of infection (Thrall *et al.* 2000), and predation (Rowe 1994), or directly through physical injury (e.g. Crudgington and Siva-Jothy 2000; Blanckenhorn *et al.* 2002), as well as through energy costs relating to male harassment (Magurran and Seghers 1994). Mating costs to females could result as a side-effect of manipulative male behaviour (i.e. pleiotropic or collateral harm), or as a direct result of male manipulation (adaptive harm), that increases the fitness of males (Morrow *et al.* 2003; Lessells 2006). Intra-sexual competition for mates can serve to inflate conflict over mating, such as in sperm competition which operates within the reproductive tract of females and beyond (Gavrilets and Hayashi 2006; Wolfner 2009). For example, in *Drosophila melanogaster*, studies have found a significant, positive, association

between male induced harm (female survival costs), caused by accessory gland proteins (Acps) transferred in the ejaculate, and defensive ability of males in sperm competition (Civetta and Clark 2000). The sex-peptide, Acp70A, is known to increase female fecundity, decrease re-mating propensity and is central to the reduction of female survival following mating, ensuring greater offspring production sired by the mating male, at the expense of future female fecundity (Wigby and Chapman 2005). Infanticide by males is another illustration of sexual conflict, which functions to induce female mating receptivity to the acting male at the expense of female fitness. Costs to females from male mating harassment can also be substantial (Magurran and Seghers 1994), and may, in the extreme, lead to convenience polyandry whereby females accept mating due to the high costs of resisting male advances (Thornhill and Alcock 1983).

Support for theory postulating that sexual conflict and subsequent SAC may function to promote population divergence and speciation is less clear however. Increased reproductive isolation among populations subjected to varied levels of sexual conflict was found in the dung fly *Sepsis cynipsea* (Martin and Hosken 2003), but not in *Drosophila* spp. (Wigby and Chapman 2006; Bacigalupe *et al.* 2007) or *Callosobruchus maculatus* (Gay *et al.* 2009), questioning the generality of SAC as a driver of intra-specific population divergence (Parker and Partridge 1998; Gavrilets and Hayashi 2005; Chapman 2006). Although the potential for sexual conflict is expected to be ubiquitous in nature, actual sexual conflict and subsequent SAC may not always be expressed (Chapman 2006). Conflict and SAC rely upon the existence of differential fitness landscapes for males and females and so divergent selection pressures. Genetic variation in traits of males and females that underlie the conflict trait (e.g. mating rate), is also necessary to allow for the possibility of (co)evolution (Chapman 2006). Indeed, female mating costs are not always apparent for promiscuous species (Arnqvist and Nilsson 2000; Martin and Hosken 2004; Reguera *et al.* 2004; House *et al.* 2008), and can vary substantially among (e.g. Rönn *et al.* 2006), and within species (Shuker *et al.* 2006). Female mating costs (conflict load) may reflect the difference in trait optima between the sexes, and if SAC acts to move the trait value closer to, or further away from, the optimal value for each sex respectively, we might expect female mating costs to fluctuate accordingly, with greater female costs apparent when mating rate is closer to the male optimum (e.g. Rice 2000).

The costs of mating will also be influenced by ecological and environmental conditions (Härdling and Kaitala 2005; Kokko and Rankin 2006), such as predation for

water striders (Rowe 1994; Arnqvist 1997; Arnqvist and Rowe 2005), frogs (Lode *et al.* 2004), and guppies (Croft *et al.* 2006; Elgee *et al.* 2010), see also (Magnhagen 1991). Additionally, habitat and/or population structure influences encounter rates between the sexes (e.g. seaweed flies, Edward and Gilburn 2007; water striders, Eldakar *et al.* 2009a; Eldakar *et al.* 2010a; see also Härdling and Kaitala 2005), and food availability is known to affect the extent of conflict in fruit flies (Chapman and Partridge 1996) and moths (Wedell *et al.* 2002b). Fundamentally the environment will determine how natural selection and sexual selection act on males and females, and so the extent to which sex specific patterns of selection conflict. Thus, selection on males and/or females could both result from, and lead to, life-history changes with further effects on mating and reproduction. For example, female guppies may seek areas of increased predation pressure to avoid male harassment (Croft *et al.* 2006; Elgee *et al.* 2010). These observations question the general importance of sexual conflict for evolution; when and how do selection forces diverge to generate sexual conflict and SAC (Chapman 2006; Wedell *et al.* 2006). Before such large questions can be assessed much empirical research is needed to ascertain where and when conflict exists in the field. Thus, an outstanding question that will further our understanding of the role of sexual conflict, concerns the expression of sexual conflict within and between closely related species across their geographic distributions.

As a step towards understanding the importance of sexual conflict in the field, I explored population variation in the expression of sexual conflict across the geographic distribution of *Lygaeus equestris* and its sister species *Lygaeus simulans* (Deckert 1985). Using six freshly caught field populations (four *L. equestris*, and two *L. simulans*), I performed common garden laboratory experiments, and considered the fitness consequences to females of elevated mating rates, and the life-history characteristics of both species. Despite being highly polygamous (Solbreck *et al.* 1989; Tadler *et al.* 1999; Shuker *et al.* 2006), previous studies of *L. equestris* have found substantial female mating costs (Sillén-Tullberg 1981) that differed among populations (Shuker *et al.* 2006). Substantial ecological and life-history differences have also been noted for populations, including predation, host-plant use, adult mass and the likelihood of multi-voltinism (Solbreck *et al.* 1989; Shuker *et al.* 2006). *L. equestris* and *L. simulans* therefore present an opportunity to empirically compare population variation in female mating costs, and relate this to life-history variation. To quantify the extent of sexual conflict over mating among populations (female mating costs), I performed three mating treatments on females from each of the six populations. Females were either: (1) mated once and retained in isolation until death; (2) retained with one male until death or; (3) retained with three males until death. I recorded the resulting

fitness consequences of each treatment, in terms of longevity, fecundity (the total number of eggs produced), and fertility (number of fertilised eggs produced). In separate experiments, I studied the life-history characteristics of each population including egg size, generation times (male and female), and adult mass (male and female). I posed three major questions in this study; (1) do populations and species vary in the extent of conflict over mating (female mating costs) experienced; (2) do populations and species differ in life-history characteristics; (3) are there patterns linking life-history variation and costs of mating across species?

Methods

Replicate populations of *Lygaeus equestris* (L)(Hemiptera: Lygaeidae) and its sister species, *Lygaeus simulans* (Deckert, 1985) were used in this study. See Chapter 2 for details of the ecology of the two species, and for general methods of bug culturing and husbandry in the laboratory.

Field population collection

Six freshly obtained populations of *Lygaeus* were used in this study, covering a wide range of their distribution (Figure 1). Two *L. equestris* populations (Morga and Geta) originated from Sweden, and were separated by a distance of approximately 200km. I sampled the Morga population from an area approximately 60 km north of Stockholm (17°38.2 E, 59°45.8 N), and the Geta population from approximately 140km south of Stockholm (16°18.1 E, 58°40.0 N). I also sampled two populations derived from the Dolomites region of northern Italy; Ledro (10°45.6 E, 45°53.7 N) and approximately 100km north east at Predazzo (11°35.0 E, 46°17.9 N, see Figure 1). The two *L. simulans* populations were sampled from two locations in the Pratomagno mountains in Saltino, Tuscany (11°32.1 E, 43°43.8 N) by Dr. David Shuker. As these Tuscan locations are separated by approximately 2 km, they likely represent repeat samples of the same overall population and I therefore refer to them as Tuscany and Tuscany2. Due to logistical constraints of finding, sampling, and maintaining populations in the laboratory, three populations (Group1: Morga, Geta and Tuscany) were sampled in June/July 2008, and three populations (Group2: Ledro, Predazzo, and Tuscany2) were sampled in the summer of 2009 (See Figure 1). The number of bugs caught and used to establish laboratory populations varied across the populations, depending on availability, from over 30 males and females for both Swedish populations, to 7 males and 7 females for the Predazzo population.

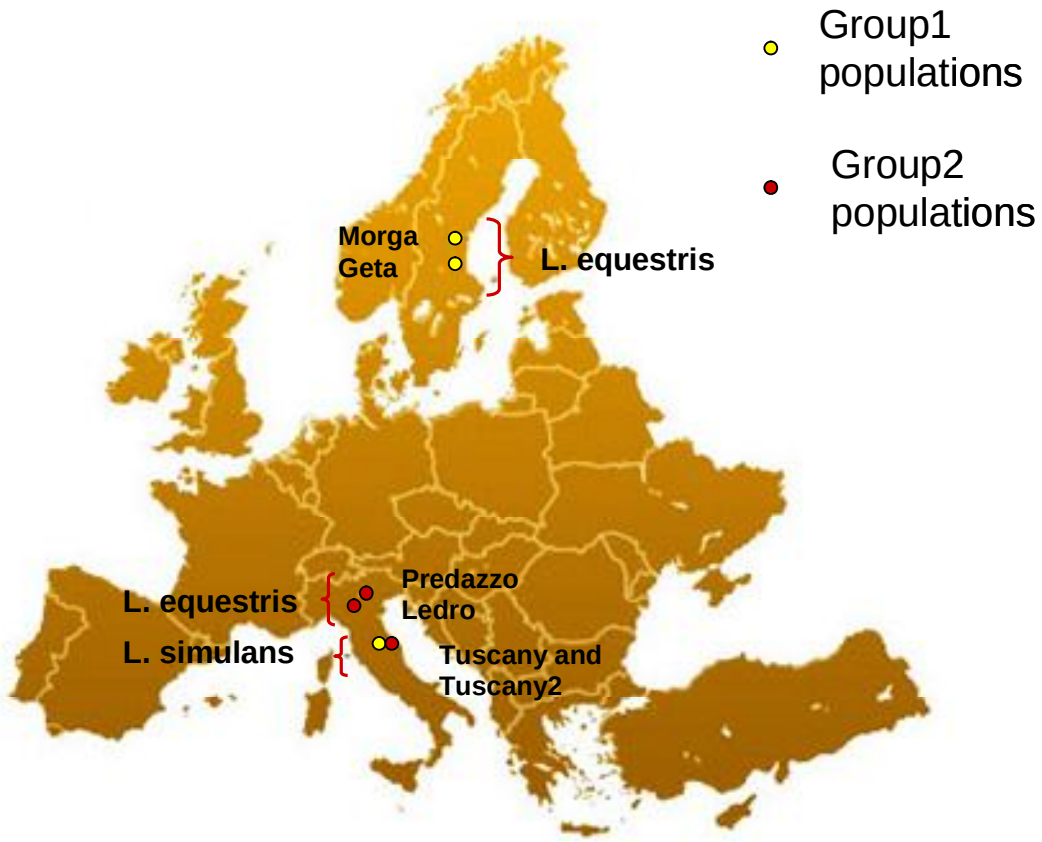


Figure 1. Diagram of population origins. Group1 populations (*L. equestris*; Morga, Geta, and *L.simulans*; Tuscany) sampled in 2008. Group2 populations (*L. equestris*; Predazzo, Ledro, and *L.simulans*; Tuscany2) sampled in 2009.

Experimental design

Female mating costs

The experiment was performed in January to May, 2009 for Group1 populations, whilst the experiment on Group2 populations was performed later, in January to May, 2010. Thus, there were two experiments, each containing two *L. equestris* populations and one *L.*

simulans population. For each population and treatment, between 20 and 27 replicate females were obtained (median = 21, total $N = 403$).

From stock cages (See Chapter 2), late instar nymphs were transferred to a large 'maturation' cage when needed. Here, freshly eclosed adults were removed every other day and placed into single sex small containers (8cm x 8cm x 5.5cm, max $N = 8$ per pot) with seeds and water, to ensure virginity and infer age for experimentation. As the number of mating events cannot feasibly be manipulated directly, due to long and variable mating durations (see Chapter 2), I used three mating treatments to test the fitness costs to females of various mating rates. Females were either: 1) mated once and retained in isolation until death (control group); 2) retained with 1 male until death or; 3) retained with 3 males until death. For treatments 2 and 3, I removed the male(s) every third day, providing an 'oviposition holiday' for females. This allowed oviposition to be unimpeded by male attention, thus restricting the risk of females becoming egg-bound (Sillén-Tullberg 1981; Sillén-Tullberg 1985b). The appropriate number of males (but not necessarily the same individuals) was returned to each female the following day (see below). To enable sufficient replication, the experiment was in the form of a rolling experiment, with males and females continuously coming through from stock cages as per availability, and placed randomly in treatments, following a period of single-sex isolation to ensure virginity prior to commencing treatments.

Virgin females were placed in their respective treatments at approximately 7 days old (6-9 days post adult eclosion). Male partners were 7-9 days old on first pairing. I carried out the treatments in transparent pots (8cm x 8cm x 5.5cm) with perforated lids containing organic sunflower seeds, and damp cotton wool each placed within small 3cm Petri dish lids or bases. For treatment 1 (control group), females were paired with a random male and scored every hour for 6 hours for copulation (stable end–end position) to ensure that copulation had occurred and where possible to ensure a single mating only. Once the pair had separated, females were retained in isolation in their pots (transferred to fresh pots every three days), and males were transferred to large 'male pool' cages (containing other males from the same respective populations) set up similarly to stock cages. As new replicate females were continually placed in treatments with 7-9 day old males, the male pool always contained a proportion of young males. Any pairs remaining in copula after this 6 hour observation period were left until they had separated before removing the male.

For treatments 2 and 3 males were removed from females every third day and transferred to male pool cages. Males from these pools were then randomly re-assigned and re-introduced to females the following day, according to treatment and population. Upon re-introduction of male(s) to females, they were placed in new pots (i.e. every third day), as were single females in treatment 1. Any eggs laid from the previous three days were then counted and isolated in a Petri dish with a piece of cotton wool only. These were retained for 5-7 days, and the number of fertilised eggs (eggs turn orange as they develop) counted. I scored replicates in treatments 2 and 3 twice daily for copulation to infer mating rates (the proportion of observations found mating). Dead males were replaced to maintain the sex ratio of the respective treatments. Thus, for each female in each treatment we recorded fecundity (total number of eggs laid, and the number of fertilised, developing, eggs produced), and longevity (days alive post treatment commencement).

Life-history experiment

20-30 females per population were each retained with one male in a small (8cm x 8cm x 5.5cm) transparent pot with a perforated lid along with organic sunflower seeds and water. Males and females were paired at least 6 days post adult eclosion to ensure sexual maturity. After 3 days of being paired any eggs oviposited were collected twice daily and transferred to small (30mm diameter) Petri dishes to develop. Collected eggs were also scanned for fertilisation from 3 days post-collection twice daily. Fertilised eggs (N = 20-35 per female) were photographed under a microscope at 2.5x magnification, and I subsequently measured the length and width of each egg using the 'ImageJ' programme (ImageJ 1.41o, NIH). After photographing, fertilised eggs were returned to their Petri dishes and scanned twice daily for hatching.

Upon hatching, same-age offspring were transferred into rearing pots (8cm x 8cm x 5.5cm) containing a layer of organic sunflower seeds (depth of ≈ 2-3 seeds) and a 7ml bijou tube (Barloworld scientific Ltd) of water, where they were retained until adult eclosion. Nymphs (from the same female) were placed in these pots at a density of 6 bugs per pot, where possible, in 4 pots and a 5th spare pot containing a maximum of 15-20 nymphs in an attempt to gain sufficient bugs reaching adulthood for analysis. Water was replaced after 10 days, or when necessary, and pots scanned daily after 20 days post-setup to record adult eclosion. Newly eclosed adults were placed in large Petri dishes (individuals of the same family that eclosed on the same day retained together) with seed and water and placed in the

incubator to melanise. After a further 24 hours these adults were placed individually in 1.5ml Eppendorfs, sexed and transferred to a freezer at -20ºC for at least 2 days. Adult bugs, retained in their eppendorfs, were then dried for 48 hours at 60ºC, before taking dry mass measurements using a mass balance to the nearest 0.0001g. For each female, I measured the width and length of each egg photographed ($N \approx 30$), and subsequently calculated egg volume as a prolate spheroid (volume = 1/6*pi*width^2*length, Solbreck *et al.* 1989). I also recorded the egg to adult development time, and corresponding adult dry mass, which could both be matched to the offspring's sex for those that developed into adults. All life-history data were averaged out to gain one value per female for each trait of interest (including egg volume, egg to adult development time for males and females separately, and adult dry mass of the sexes separately). In total 3,893 eggs from 150 females were photographed and measured, (mean = 26 eggs per female, and mean = 25 females per population). For data on subsequent development of offspring, a total of 3,554 offspring reached adulthood from 154 females. I therefore obtained data from 22-30 (median = 24) females from each population. Due to the occurrence of some single sexed broods, the total number of females that produced males and females differed slightly (*N* producing females = 154, *N* producing males = 148).

Statistical analysis

I used a combination of general linear model and traditional ANOVA approaches for the analyses. Full models were built and terms tested when fitted last in the model respective of hierarchy in R (R version 2.11.1) to determine the significance of terms. For the general linear models, likelihood ratio chi-squared tests, or *F* tests where necessary (i.e. when quasi-likelihood error structures were employed), were performed to test for deviance between models (Crawley 2007). Non-significant interaction effects were reported, removed, and models containing only the main effects were retested.

As Group1 and Group2 populations were experimented upon in different years, they were always analysed separately. I first performed analyses on *L. equestris* populations only. Thus, for these intra-specific analyses, one set of analyses compared the Geta and Morga populations (i.e. Group1), while a second set of analyses compared the Ledro and Predazzo populations (Group2). To gain insight into inter-specific patterns, these analyses were then repeated, but each group was expanded to include its respective population of *L. simulans.*

Mating rate and mating costs

Mating rate (proportion of observational scans found mating) was analysed as a logistic analysis, fitting a GLM with quasi-binomial error structure and a logit link, to account for overdispersion of the data. To investigate the fitness consequences to females of elevated mating rates, I calculated a suite of fitness proxies including female longevity, fecundity (total number of eggs), fertilisation success (proportion of eggs fertilised) and fertility (the number of fertilised eggs produced). Each of these fitness measures were tested against fixed effects of treatment and population and their interaction.

Cox's proportional hazards survival analysis was employed to investigate longevity. Due to the presence of zeros in the fecundity data (some females not laying eggs), fecundity was analysed firstly using a binary logistic analysis (GLM with a binomial error structure and logit link) of egg presence/absence against treatment and population. For those females that did oviposit eggs (reproductive females), fecundity was analysed as the number eggs produced (square root transformed) using ANOVA.

Fertilisation success (i.e. the proportion of eggs fertilised) of reproductively active females was analysed with a logistic analysis (GLM with quasi-binomial error structure and logit link). Fertility (fertilised egg production) was analysed in the same manner as for fecundity of reproductive females. I performed a binary logistic analysis on whether fertilised (developing) eggs were laid or not. I then used ANOVA on the number of fertilised eggs (square root transformed) laid by those females that laid fertilised eggs.

Life history

To examine population variation in baseline life-history traits, control group (once mated) females from the mating costs experiment were used to explore population differences in survival, fecundity and fertility (using either GLMs with the appropriate error structures, or ANOVAs of transformed data). For the life history experiment, data from the offspring of the same female (i.e. siblings) were averaged together to avoid pseudoreplication. Subsequently I analysed egg volume across populations as a one way ANOVA. Egg to adult development time and adult dry mass measures were calculated for each sex within female. A factorial ANCOVA of development time against population and sex was performed

controlling for the mean egg size of each female (i.e. development time ~ population*sex + mean egg size, NB the eventual sex of eggs could not be determined). Similarly, adult dry mass was analysed with a factorial ANCOVA against population and sex controlling for egg size. For these analyses an interaction term between population and egg size was added after all others to test for population differences in the effect of egg size on development time and dry mass. Finally, factorial ANCOVAs of adult dry mass against development time, population and sex were performed to see how dry mass associated with development time among the respective populations.

Results

Female mating costs

Intra-specific analyses of *L. equestris*

Mating rate

The experimental treatments were successful in changing the proportion of observational scans where pairs were found to be mating (henceforth described as mating rate) in the expected direction (Figure 2). There was a significant interaction between population and treatment on mating rate in Group1 populations (interaction; $F_{1,79}$ = 4.72, p = 0.033, treatment effect; $F_{1,80}$ = 35.16, p < 0.0001, population effect; $F_{1,80}$ = 7.03, p = 0.01), largely driven by females from the Morga population displaying a lower increase in mating rate over the two treatments (Figure 2). There was no such interaction among Group2 populations ($F_{1,88}$ = 2.5 x10^{-6}, p = 0.999), where the populations did not differ significantly in their overall mating rates ($F_{1,89}$ = 2.954 p = 0.089), and only treatment was significant ($F_{1,89}$ = 80.935 p < 0.001; Figure 1).

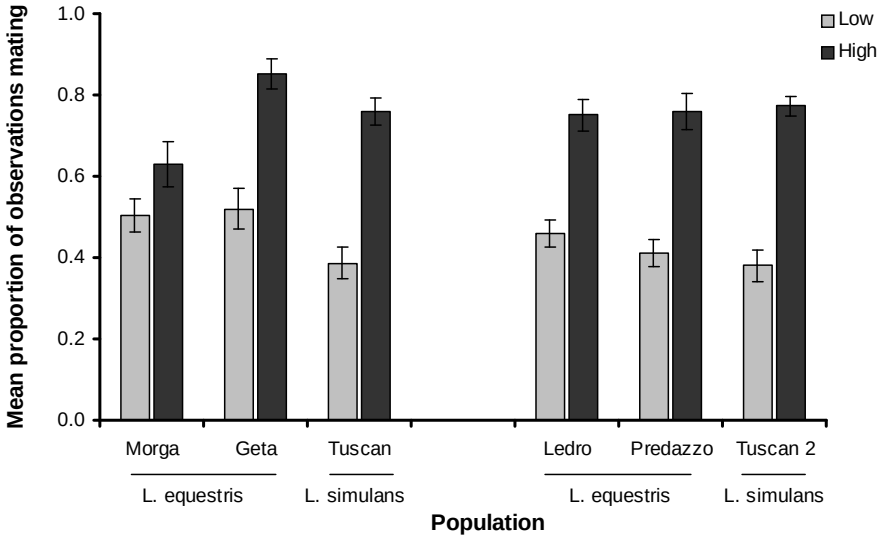


Figure 2. Mean proportion of observational scans found mating for the low (1:1 sex ratio, light grey) and high (3m:1f sex ratio, dark gray) mating treatments for each population respectively. Populations are split by the experimental groups in which they were performed (Group1 left, Group2 right). Within each experimental group two *L. equestris* populations and one *L. simulans* population were studied respectively. Error bars are standard errors.

Longevity

Exposure of females to males (i.e. treatment) reduced female longevity across all populations (Figure 3A,B; Group1; $\chi^2 = 41.55$, $df = 2$, $N = 124$, $p < 0.001$, Group2; $\chi^2 = 45.49$, $df = 2$, $N = 138$, $p < 0.001$), with longevity reduced by as much as 45 days in the high mating treatments (Figure 3A). There was no difference between populations in their overall longevity in either group (Group1; $\chi^2 = 1.86$, $df = 1$, $N = 124$, $p = 0.173$, Group2; $\chi^2 = 1.41$, $df = 1$, $N = 138$, $p = 0.235$), nor was there a treatment by population interaction on longevity (Group1; $\chi^2 = 0.248$, $df = 2$, $p = 0.883$, Group2; $\chi^2 = 0.239$, $df = 2$, $p = 0.887$, Figure 3A), suggesting there to be no population difference in the magnitude of survival costs associated with increased female mating rates.

Fecundity

Amongst the Group1 populations, fewer females in the high mating treatment produced eggs (i.e. were reproductive) than in either the control or low mating treatments (Table 1A and 1B. However, there was no difference between the treatments in the proportion of females producing eggs among the Group2 populations, and there were no population, or interaction effects between treatment and population on the proportion of females producing eggs in either region (see Table 1A).

Overall, the number of eggs produced (by reproductive females) differed across treatments in both regions. For reproductive females (Group1: $N = 95$, Group2: $N = 113$), significantly fewer eggs were produced from females in the high mating treatments for both regions compared with the other treatments (Group1; $F_{2,91} = 3.78$, $p = 0.026$, Group2; $F_{2,109} = 7.300$, $p = 0.001$, see Figure 3B). Females from the Group1 populations did not differ in the number of eggs produced overall ($F_{1,91} = 0.080$, $p = 0.778$). However, within Group2, the Predazzo females produced significantly more eggs than Ledro females ($F_{1,109} = 14.695$, $p < 0.001$). For both regions, the treatments did not affect populations differently in terms of the number of eggs produced (interaction effect; Group1; $F_{2,89} = 0.032$, $p = 0.969$, Group2; $F_{2,107} = 0.761$, $p = 0.470$; Figure 3B). Thus, there was no evidence of population differences in the effect of treatment on fecundity, and hence differential fecundity costs of increased mating among the populations was not observed.

Fertility

The proportion of eggs fertilised (i.e. that commenced development) was significantly affected by treatment in both groups (Group1: $F_{2,91}$ = 35.203, p <0.001, Group2; $F_{2,109}$ = 12.952, p <0.001), with a substantially lower proportion of eggs fertilised amongst once mated females (see Figure 4A). This was the only difference for the proportion of eggs fertilised however (population effects: Group1: $F_{1,91}$ = 0.009, p = 0.925, Group2; $F_{1,109}$ = 0.566, p = 0.453. Interaction effects: Group1; $F_{2,89}$ = 0.077, p = 0.926, Group2; $F_{2,107}$ = 0.049, p = 0.952, Figure 4A).

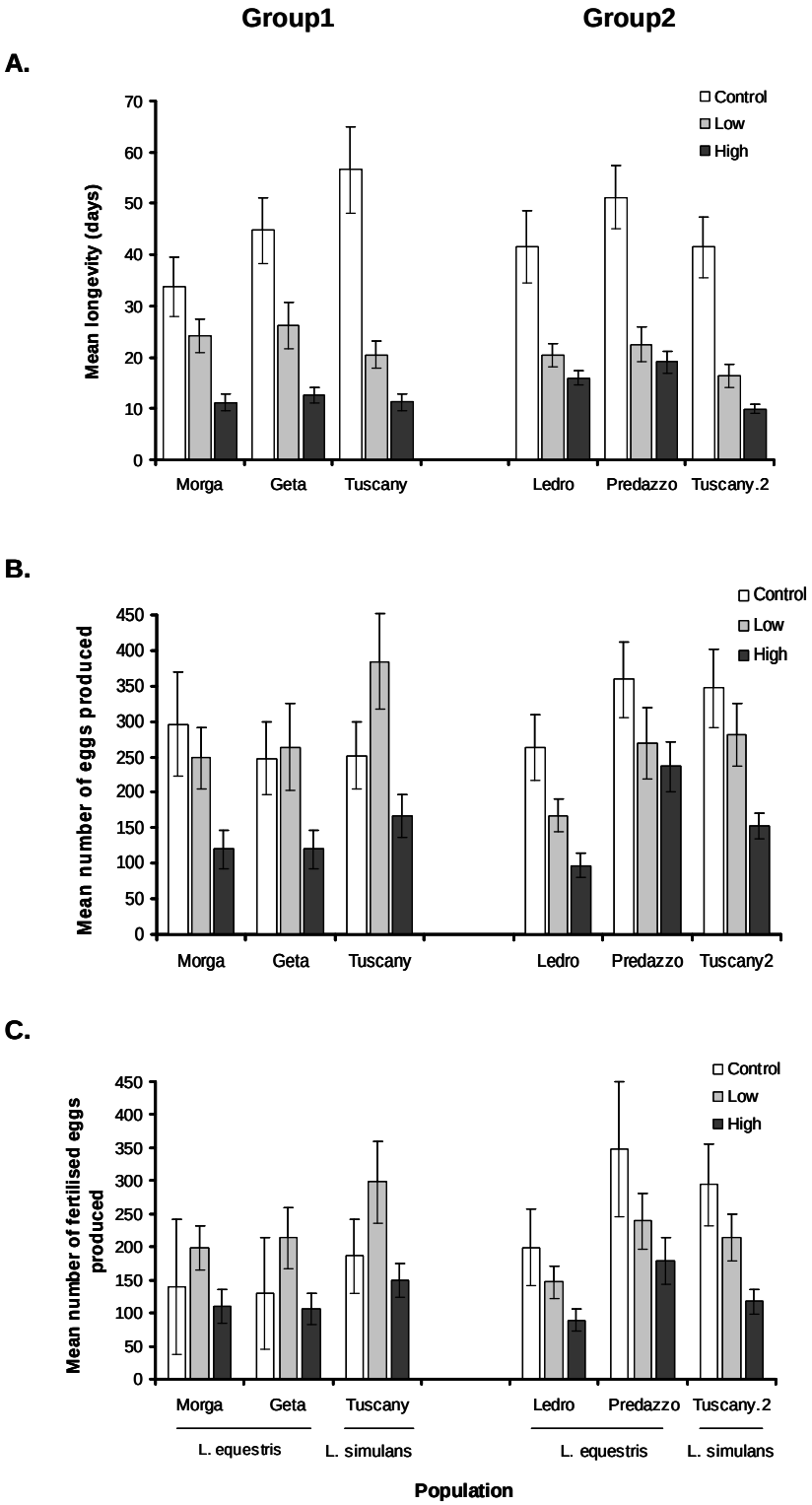


Figure 3. The fitness consequences against treatment for each population in terms of (A) mean longevity, (B) fecundity (number of eggs oviposited by reproductive females), and (C) number of fertilised (developing) eggs produced (by fertile females). The population, and species, origins are as indicated, and are clustered according to experimental groups. Error bars are standard errors. See text for details.

Table 1A. Binary logistic analysis of egg presence within *L. equestris* females of Group1 (*N* = 124) and Group2 (*N* = 138) against the fixed effects of treatment and population. Df is degrees of freedom. LR χ^2 is the likelihood ratio Chi-squared deviance. Significant p-values are shown in bold.

	Group 1			Group 2		
Source	***Df***	**LR χ^2**	***p***	***Df***	**LR χ^2**	***p***
Treatment	2	13.632	**0.001**	2	0.097	0.953
Population	1	3.177	0.075	1	0.396	0.529
treatment*population	2	2.512	0.285	2	2.372	0.306

Table 1B. Effect sizes of factor levels in the reduced model for Group1 populations. Intercept is the mean for females from Geta population control (once mated) treatment group. All coefficients thereafter are treatment contrasts. SE is standard error for the coefficient respectively. Coefficients are in logits due to the use of a binomial error structure suitable for a binary response variable. Significant effects of the z score highlighted with asterisks.

Model: egg presence~ treatment+ population, binomial

Source	**Estimate coefficient**	**SE**	**Z (sig)**
Intercept	2.22	0.534	4.155 (***)
Treatment: High	-1.532	0.551	-2.78 (**)
Treatment: low	0.234	0.656	0.356
Population: Morga	-0.816	0.466	-1.751

In both regions, the proportion of reproductive females producing fertilised eggs varied across treatments. Once mated females showed a significantly lower incidence of fertilised (and developing) egg production (Group1: LR χ^2 = 29.886, *df* = 2, $p < 0.001$, Group2; LR χ^2 = 26.418, *d.f.* = 2, $p < 0.001$, Figure 4B). However, within the groups, the populations did not differ in the incidence of fertilised egg production (Group1: LR χ^2 = 0.035, *df* = 1, $p = 0.852$, Group2: LR χ^2 = 0.699, *d.f.* = 1, $p = 0.403$, see Figure 4B), and no interaction effects between population and treatment on the proportion of females producing

fertilised eggs were found (Group1: LR χ^2 = 0.239, *d.f.* = 2, p = 0.887, Group2; LR χ^2 = 4.819, df = 2, p = 0.090).

There was no consistent pattern between the two groups, in terms of the number of fertilised eggs produced (by females that produced fertilised eggs, *N*: Group1 = 69, Group2 = 94). For Group2, Ledro females produced significantly fewer fertilised eggs overall than Predazzo females (population effect; $F_{1,90}$ = 9.140, p = 0.003), but no population effects were apparent in Group1 populations ($F_{1,65}$ = 0.009, p = 0.925, Figure 3C). Conversely, treatment had a significant effect on the number of fertilised eggs produced in Group1 *L. equestris* ($F_{2,65}$ = 4.136, p = 0.020), with the high mating treatments consistently displaying fewer fertile eggs, but this was not the case among the Group2 populations ($F_{2,90}$ = 2.290, p = 0.107, Figure 3C). Within both groups however, there were no interaction effects between treatment and population on the number of fertilised eggs produced (Group1; $F_{2,63}$ = 0.026, p = 0.975, Group2; $F_{2,88}$ = 0.026, p = 0.974, see Figure 3C). Therefore, as with the longevity and fecundity analyses above, there was no evidence that populations differed in the fitness costs of increased mating in terms of the number of fertilised eggs produced. Direct analyses of mating rate rather than the treatments per se, against the fitness proxies revealed the same qualitative results as shown above (data not shown).

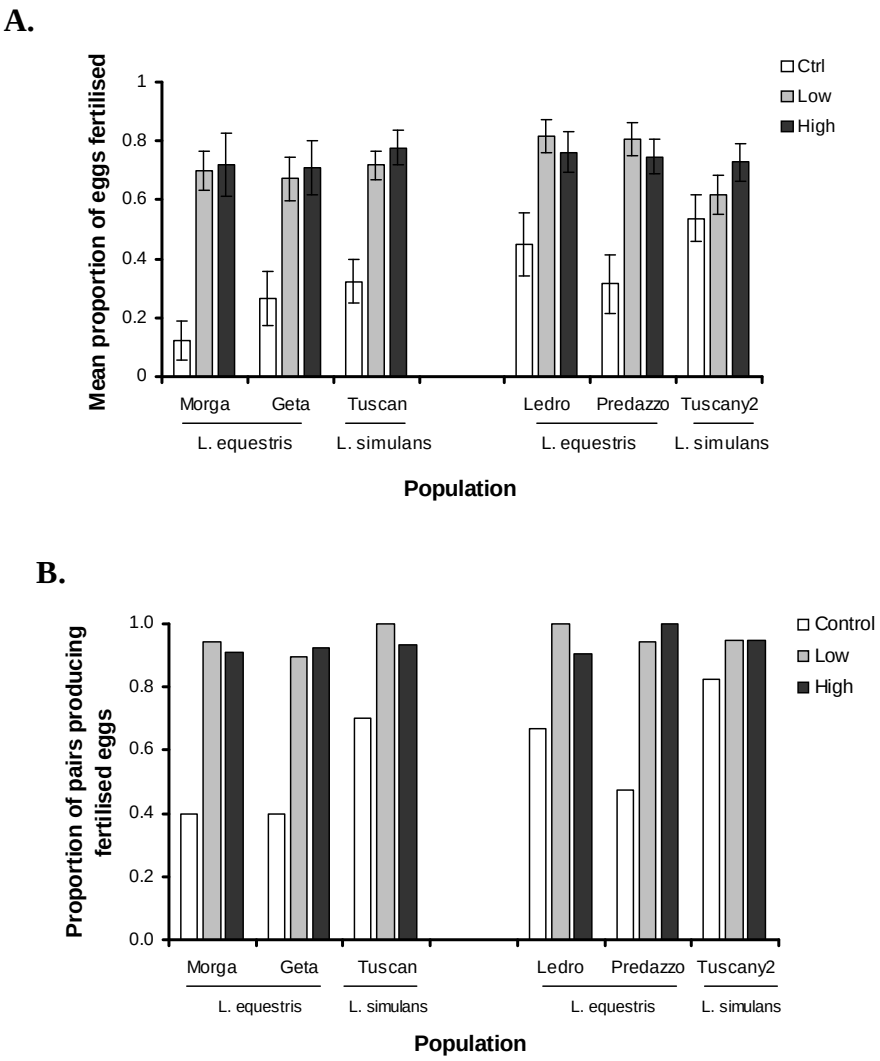


Figure 4. Fertilisation success (of reproductive females) for each mating treatment and population. (A) the mean proportion of eggs oviposited, and commencing development. (B) the proportion of females producing fertilised eggs. Populations are split by experimental group, and the species are as indicated. Error bars for in A are standard errors.

Inter-specific analyses

Expanding the analyses to include *L. simulans* populations from the Tuscany region that were tested concurrently with *L. equestris* from Sweden (Group1) and from the Dolomites of Northern Italy (Group2) respectively, largely conformed to the results gained from the intra-specific *L. equestris* analyses above. Thus, here I report only briefly on traits for which *L.*

simulans responded similarly to *L. equestris*, providing more detail for cases where the inclusion of *L. simulans* changed the results qualitatively. *Lygaeus simulans* displayed lower survival overall in experimental Group2 (population effect: χ^2 = 10.476, N = 215, *d.f.* = 2, p = 0.005), but this species difference was not apparent in Group1 (population effect: χ^2 = 1.938, N = 188, *d.f.* = 2, p = 0.38, Figure 3A). Although the data suggest there may be a species difference in the effect of treatment on fecundity amongst Group1 populations, with Tuscany females perhaps benefiting from low mating rates (Figure 3B), including *L. simulans* in the fecundity analyses yielded no difference in qualitative outcomes to when *L. equestris* were analysed on their own. The significant treatment effects on the number of eggs produced remained for both groups (Group1; $F_{2,143}$ = 5.193, p = 0.007, Group2; $F_{2,171}$ = 9.453, p < 0.001), and population was only significant in Group 2 (Group1; $F_{2,143}$ = 1.023, p = 0.362, Group2; $F_{2,171}$ = 7.974, p < 0.001), with no interaction effects in either group (Group1; $F_{4,139}$ = 0.373, p = 0.828, Group2; $F_{4,167}$ = 0.852, p = 0.494, see Figure 3B). For the proportion of eggs fertilised, a population effect was found in Group1 ($F_{2,143}$ = 4.634, p = 0.011), with Tuscany *L. simulans* displaying greater fertilisation success overall than Morga *L. equestris* females, but such a population effect was not found in Group2 ($F_{2,171}$ = 0.797, p = 0.452), suggesting there to be no inherent difference between the two species in fertilisation success (Figure 4A). However the interaction effect between population and treatment on fertilisation success was only marginally non-significant in Group2 ($F_{4,167}$ = 2.350, p = 0.056, Figure 4A). Additionally, the proportion of reproductive females producing fertilised eggs was only marginally non-significant between the populations in Group1 (LR χ^2 = 5.862, *d.f.* = 2, p = 0.053, Figure 4B).

Life history

Intra-specific analyses of *L. equestris*

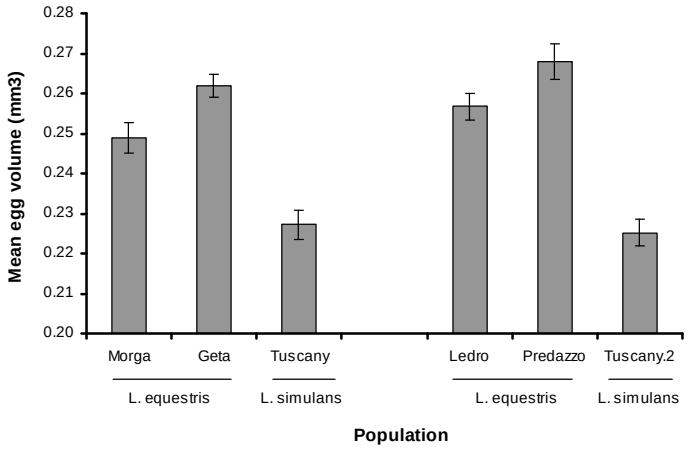
For *L. equestris*, there was no population variation in longevity, fecundity (in terms both the incidence - and the number - of eggs produced), and fertility (in terms of incidence – and number – of fertilised eggs produced) of once mated females in the mating cost experiments, within either region (Table 3). However, egg volume differed between the *L. equestris* populations in both groups (Group1: $F_{1,49}$ = 8.013, p = 0.007, Group2; $F_{1,50}$ = 4.299, p = 0.043, Figure 5A), as did egg to adult development time controlling for egg size and sex in Group1 ($F_{1,98}$ = 124.4, p < 0.001). Group2 populations did not differ in development time however ($F_{1,98}$ = 1.793, p = 0.184, Figure 5B). Within both Groups, there was no difference

in the development times of males and females respectively (Group1: $F_{1,98}$ = 2.265, p = 0.136, Group2: $F_{1,98}$ = 1.459, p = 0.230), nor was there a sex by population interaction (interaction effect: Group1; $F_{1,97}$ = 0.018, p = 0.892, Group2; $F_{1,97}$ = 0.003, p = 0.961, see Figure 5B). However, including egg volume as a covariate revealed that the relationship between egg size and development time differed in Group2 populations (interaction effect: $F_{1,97}$ = 4.607, p = 0.034, Figure 6B), but not among the Group1 populations ($F_{1,97}$ = 1.472, p = 0.228, Figure 6A). Specifically, the Ledro population showed a negative relationship whilst Predazzo bugs showed no obvious relationship (Figure 6B).

Table 3. Population variation in baseline fitness traits (using once mated females) of the *L. equestris* populations. Longevity analysed as survival analysis. Fecundity was analysed in terms of the incidence of females producing eggs and the number of eggs produced (log transformed) by the subset of females that did produce eggs. Fertility was analysed in terms of the incidence of fertilised egg production among females that oviposited, and also the number of fertilised eggs (Group1; log transformed, Group2; square root transformed) produced by females that produced fertilised eggs. *N* = number of replicates in each analysis, *D.f.* is degrees of freedom, the test statistic is either likelihood ratio Chi-squared test (for GLMs) or *F* ratio (for ANOVAs).

			Group 1				Group 2	
Trait	***N***	***D.f.***	**Test stat**	***P***	***N***	***D.f.***	**Test stat**	***P***
Longevity:			χ^2				χ^2	
Survival	41	1	0.838	0.36	46	1	0.052	0.819
Fecundity:			χ^2				χ^2	
Incidence	41	1	3.603	0.058	46	1	2.621	0.105
			F				*F*	
Number of eggs		1,33	0.042	0.839		1,35	2.270	0.141
Fertility:			χ^2				χ^2	
Incidence	35	1	0	1	37	1	1.414	0.234
			F				*F*	
Number of fertilised eggs		1,12	0.722	0.412		1,19	1.078	0.312

A.



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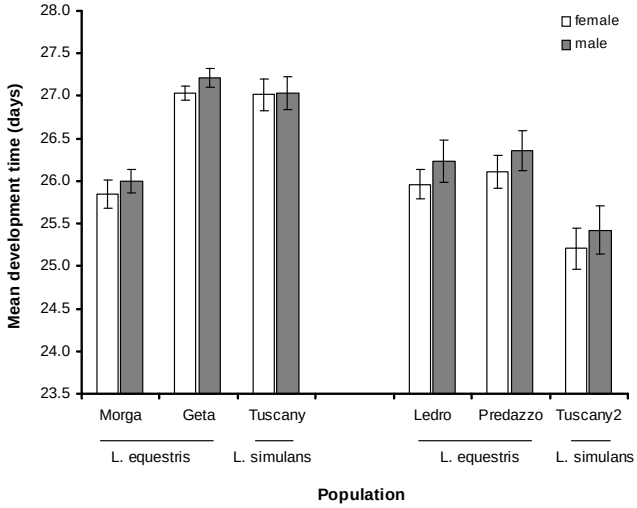

C.

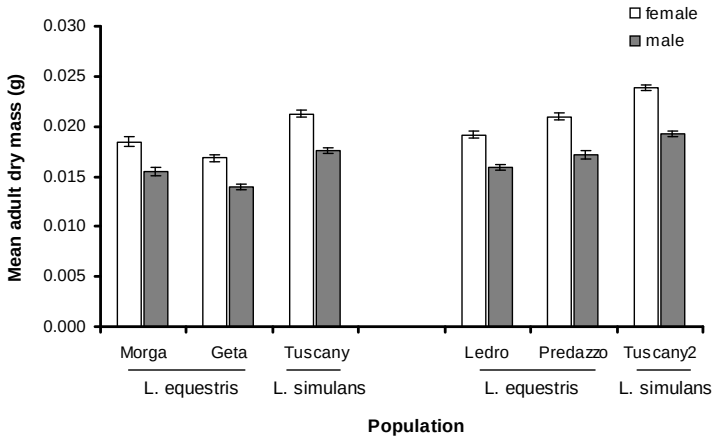

Figure 5. Life history characters for each population. A) Mean egg volume (mm^3). (B) Mean egg to adult development time (days) split by sex for each population. C) Mean adult dry mass (g) split by sex for each population. Error bars are standard errors. Populations are split by experimental group and species as indicated.

In both regions, the *L. equestris* populations differed in adult dry mass (Group1: $F_{1,98}$ = 18.280, $p < 0.001$; Group2: $F_{1,98}$ = 14.521, $p < 0.001$), as did the sexes (Group1: $F_{1,98}$ = 79.020, $p < 0.001$; Group2: $F_{1,98}$ = 105.878, $p << 0.001$, Figure 5C), but this sex effect was consistent across populations (Group1 interaction: $F_{1,97}$ = 0.042, p = 0.837, Group2: $F_{1,97}$ = 0.607, p = 0.438, Figure 5C). Generally, egg volume was not associated with final adult dry mass (Group1; $F_{1,98}$ = 0.062, p = 0.804, Group2; $F_{1,98}$ = 1.015, p = 0.316, Figure 6C and 6D), however, the Group2 populations varied in this respect (interaction effect; $F_{1,97}$ = 5.729, p = 0.019, Figure 6D).

Finally, I tested for associations between development time and dry mass controlling for sex and egg size. There was no association (though only marginally non-significant) between development time and adult dry mass in Group1 ($F_{1,98}$ = 3.186, p = 0.054), and none of the respective interaction terms (between each of development time, population, and sex) were significant for dry mass (all $p > 0.5$, see Figure 6E). However, in Group 2, development time showed a strong negative relationship with adult dry mass ($F_{1,104}$ = 23.668, $p << 0.001$), but, again, none of the respective interaction terms were significant (all $p > 0.13$, see Figure 6F).

Inter-specific analyses

As with the mating costs experiments above, extending the analyses of life-history traits to include *L. simulans* populations, tested concurrently with *L. equestris* in Group1 and Group2 respectively, largely conformed to the results gained from the intra-specific *L. equestris* analyses above. In both groups *L. simulans* eggs were smaller than those of *L. equestris*, reflected in the further increased significance of the population effect on egg volume (group1: $F_{2,73}$ = 29.140, $p < 0.001$; group2: $F_{2,71}$ = 32.708, $p < 0.001$, Figure 5A). Baseline life history characters (from once mated females in the mating costs experiments), suggest that *L. equestris* exhibit lower survival rates (Group1 population effect; N = 65, $LR\ \chi^2$ = 7.146, p = 0.028). Such a species difference was not found in Group2 populations however, and contrasts with previous results when all treatments are considered together (Figure 3A). Development time, controlling for sex and egg size, was faster in *L. simulans* than *L. equestris* in Group2 (population effect; $F_{2,133}$ = 7.365, $p < 0.001$, Figure 5B and 6B). This was not the case in Group1, and the difference in development time between the two Swedish *L. equestris* populations was similar in magnitude to the difference observed between the two species in Group2 (Figure 5B). However, the relationship between egg size and development time differed among the populations in both groups (Group1: $F_{2,138}$ =

3.379, $p = 0.037$, Group2: $F_{2,131} = 7.321$, $p < 0.001$), with *L. simulans* populations showing a positive relationship, whilst the relationship tended to be negative in form for *L. equestris* populations (see Figure 6 A and B). Despite *L. simulans* being heavier as adults in groups

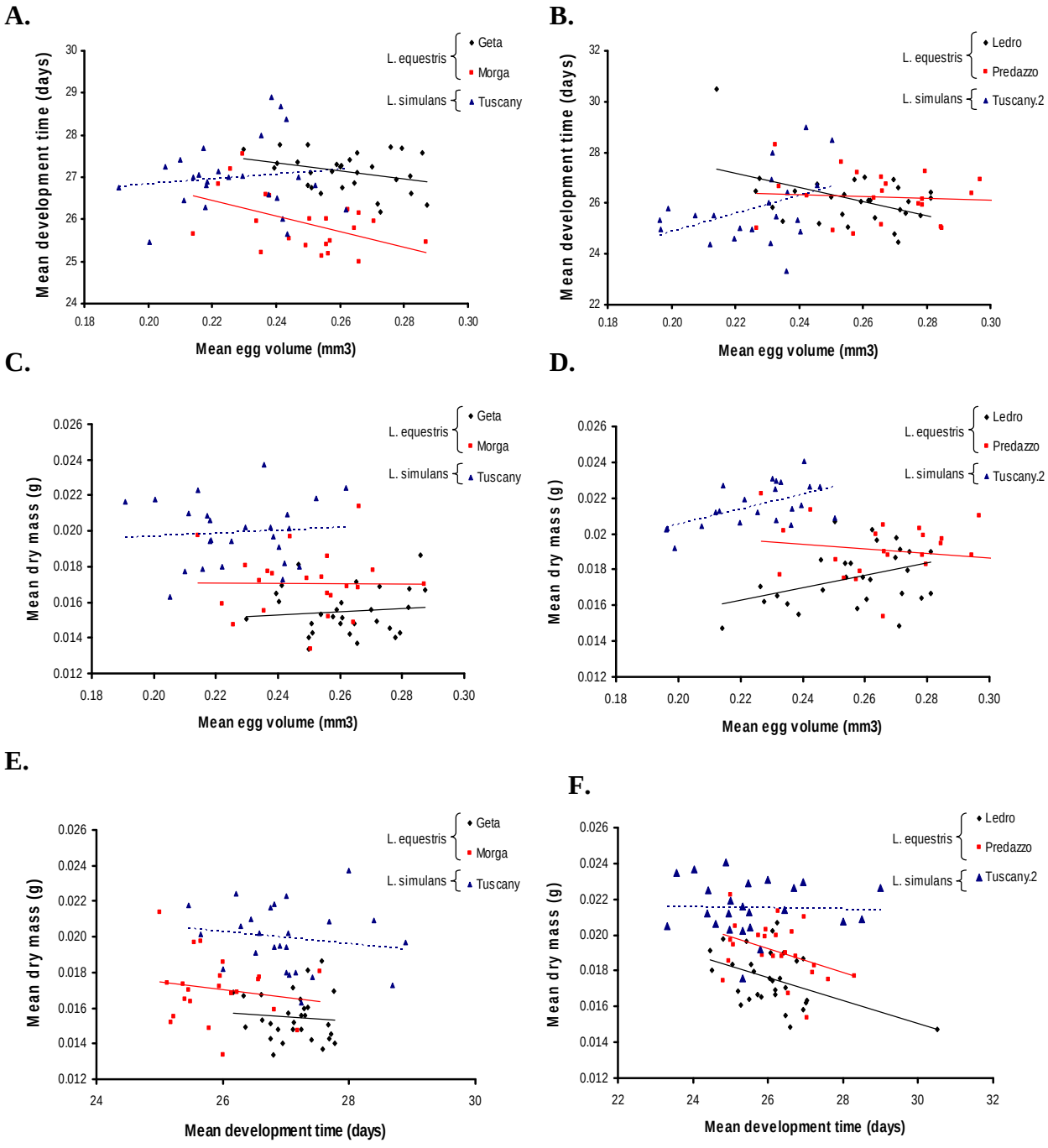

Figure 6. Life history correlations using family means for each population. (A) and (B) Show development time against egg volume, C and D) Adult dry mass against egg volume, E and F) Adult dry mass against development time. Graphs to the left side of the panel (A, C, E) show Group1 populations, those to the right (B, D, F) are Group 2. *L. simulans* highlighted with triangles and dashed lines.

(Figure 5C) there was no apparent species difference in the relationship between egg size and eventual adult dry mass (Fig 6C and 6D). For Group2 populations, there was a significant difference between the species in the effect of development time on eventual adult dry mass (interaction effect: $F_{2,140} = 3.611$, $p = 0.030$), with *L. equestris* bugs showing a negative associations and *L. simulans* showing little association (see Figure 6F). This was not the case in Group1 populations however (Figure 6E).

Results summary

I found large survival costs from increased mating, as well as differential development time, egg size, and adult mass among the Swedish (Group1) *L. equestris* populations. Amongst the Italian (Group2) *L. equestris*, I also found large survival costs from the mating treatments, as well as large costs in terms of the number of eggs and the number of fertilised (developing) eggs produced. These two populations also varied in the number of eggs produced and the number of fertilised eggs produced overall, with reproductive Predazzo females produced more, larger eggs, and more fertilised eggs, than Ledro females. Indeed Predazzo females were also heavier than Ledro females upon adult eclosion and this may explain these fecundity differences. *Lygaeus simulans* produced smaller eggs than *L. equestris* but were in fact heavier as adults suggesting a different developmental schedule than that exhibited by *L. equestris*. Perhaps the most notable potential difference between the species concerns aspects of sperm transfer and fertilisation however, as the data suggest that *L. simulans*, may be better able to ensure successful sperm transfer and fertilisation than *L. equestris*.

Discussion

Theory predicts sexual conflict to be rife among sexually reproducing species and, in the potential for sexually antagonistic co-evolution, it may be an important driver of population divergence and evolutionary change within and between species (Parker 1979; Parker and Partridge 1998; Rice 2000; Martin and Hosken 2003; Arnqvist and Rowe 2005). However, how sexual conflict varies across environments and therefore differing selection regimes is uncertain (Härdling and Kaitala 2005; Chapman 2006). Put simply, across populations do the selective optima of traits for males and females vary a lot or only a little? Here we explored sexual conflict over mating in terms of the magnitude of female mating costs across populations and between species sampled from the field in common garden laboratory experiments. I also compared life history variation amongst the populations. Generally the results suggest that female mating costs can be large, and that life history characteristics vary both within and among the species. No clear associations between mating costs and life-history measures were apparent, albeit given the limited number of populations examined. The clearest results confirm that fitness costs to females from elevated mating rates can be large, but that different fitness measures (longevity, lifetime egg production and lifetime fertile egg production) can give different results (Arnqvist and Nilsson 2000; Hunt *et al.* 2004b), and that repeated mating may, up to a point, be beneficial to females for fertilising eggs (Figure 4A and 4B, e.g. Tuscany *L. simulans* in Group1; Figure 3B and C).

As with a previous study (Shuker et al 2006) the low and high mating treatments successfully manipulated female mating rate (the proportion of observations found in copula) in the expected direction. The mating rate for females should therefore have been moved away from the female optimum in all six populations. The results clearly demonstrate large female costs to increased levels of mating (and male harassment) in *Lygaeus*, as found in two populations studied previously (Shuker *et al.* 2006). Shuker and colleagues (2006) found that females retained with three males lived 41.4% and 51% as long as once mated females of the respective populations, producing 12.9% and 37.8% as many eggs. Here I found female longevity further reduced, ranging from 20% to 38% of once mated females when housed with three males, of the respective populations, and producing between 36.9% and 66.4% as many eggs. Of those females that produced fertilised eggs, females retained with three males produced between 40% and 82.6% as many fertilised eggs as once mated females. The use of temporal female refuges in this study, may account for some of the differences, yet from

both experiments it is apparent that female mating costs can be large in Lygaeidae (see also Sillén-Tullberg 1981).

The cause of these costs remain unknown but likely include costs derived from accessory proteins transferred in the male ejaculate, as are common among insects (Chapman *et al.* 1995; Wigby and Chapman 2005; Wolfner 2009) and have recently been shown in the lygaeid *Togo hemipterus* functioning to reduce female re-mating (Himuro and Fujisaki 2008). Physical injury during copulation (Crudgington and Siva-Jothy 2000), infection risk (Thrall *et al.* 2000), and energetic costs of the prolonged copulations in these species (Kugelberg 1973b; Sillén-Tullberg 1981; Shuker *et al.* 2006), could also contribute to these costs. Male harassment may also be present in the mating costs described here, and can be substantial (e.g. in guppies Magurran and Seghers 1994). However, previous studies have found that mating itself is costly in Lygaeidae (e.g. Shuker *et al.* 2006; Himuro and Fujisaki 2008), and although harassment by males rendered unable to mate, did reduce female fitness, the magnitude of this cost was much less than that incurred by females when mating was allowed (Shuker *et al.* 2006). Indeed, despite incorporating periodic female refuges from males, female mating costs here were analogous to when females were retained without any such refuge (Shuker *et al.* 2006). Similarly, in *Drosophila* allowing females a spatial refuge did not alleviate mating costs significantly even though it did reduce female mating rates (Byrne *et al.* 2008). However, here mating rate was measured as the proportion of scans that a female was found mating. Thus it may not reflect number of mating events *per se*, but rather mating duration. In the presence of three males it may pay females to remain in copula with one male for a prolonged period rather than repeatedly being harassed and mated (convenience polyandry, Thornhill and Alcock 1983; Arnqvist and Rowe 2005).

As noted above, the fitness costs of elevated mating rates, and the way costs (benefits) of mating accrue, differed depending on the fitness trait considered. These differences highlight the importance of considering multiple aspects of fitness to reliably interpret fitness consequences associated with experimental treatments (Arnqvist and Nilsson 2000; Hunt *et al.* 2004b). For example, in both regions, mating appears to reduce female longevity in a dose dependent manner, whilst some degree of multiple mating may not be detrimental, and may even be beneficial to females in terms of fecundity and fertility, which are likely to be a better indication of fitness than survival alone (Arnqvist and Nilsson 2000; Hunt *et al.* 2004b). I found no evidence that the magnitude of mating costs varied within *L. equestris* or between the two species, suggesting there to be little difference in sexual

conflict among populations. However field populations may still vary in the extent of sexual conflict over mating, as only six populations, in two sets of three and pairs of *L. equestris*, were studied here, yielding low power to detect any such differences.

As is the case in other insects, and contrary to the two populations studied by Shuker *et al* (2006), the results here suggest that some degree of multiple mating may be beneficial to females of some populations, and thus the nature of costs and how they accrue may well differ across populations in the field (reviewed by Arnqvist and Nilsson 2000). Possible reasons for this include avoiding sperm limitation or mate incompatibility (Arnqvist and Nilsson 2000; Simmons 2005). This would concur with the general context of low fertilisation success for once mated females relative to low and high mating treatments as found here. Alternatively, multiple mating, and more specifically polyandry, may be important in protecting against selfish genetic elements, including sex ratio meiotic drive, as has been found in *Drosophila pseudoobscura* (e.g. Price *et al.* 2010). Other direct benefits from mating, such as water and nutrient transfer in the ejaculate (Arnqvist and Nilsson 2000; Simmons 2005), are less likely as an explanation here as the number of eggs oviposited by once mated females (that oviposited) was generally greater than, or equivalent to, those of females in the low mating treatment. In the field however, where environmental conditions may vary, food availability may be unpredictable (Solbreck and Sillén-Tullberg 1990; Tullberg *et al.* 2000) and such effects may become important (Wedell *et al.* 2002b).

There were some population differences in the proportion of eggs fertilised. As such, the populations may differ in male sperm transfer ability, female sperm storage, the compatibility of mates or the action of cryptic female mate choice (reviewed by Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Simmons 2005) as has been suggested to occur mechanically (through genital morphology) in *L. simulans* (Micholitsch *et al.* 2000). Cryptic female mate choice may seem unlikely given that the once mated females did not have the opportunity to trade-up sperm on encountering a better male. However, this does not preclude mate choice itself functioning to avoid mating low quality/incompatible males in the first instance (Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Simmons 2005). Shuker *et al* (2006) also found evidence of sperm limitation in *L. equestris*, and sperm transfer failure from single matings is highly apparent in *L. simulans*. Tadler *et al* (1999) found approximately 30% of male mating attempts failed, and only 60% of copulations led to successful insemination of females (Tadler *et al.* 1999; Micholitsch *et al.* 2000). Among the populations tested here, 40%-80% of once mated females (that laid eggs) laid fertilised

eggs, suggesting similar levels of sperm transfer failure in our *L. simulans* populations and even greater sperm transfer failure among *L. equestris* populations. Indeed, insemination failure is likely to be far more common in nature than described (Eberhard 1996), and along with the complex genital morphology of Lygaeidae bugs (Bonhag and Wick 1953; Tadler 1999; Tadler *et al.* 1999; Micholitsch *et al.* 2000; Huber *et al.* 2007) this suggests that there is scope for sexual selection here as well as sexual conflict over insemination and sperm use. Also noteworthy however, is that the production of unfertilised eggs may represent 'trophic eggs' that function to provide nourishment for young, and may be an adaptive strategy to unpredictable resource availability (e.g. Kudo and Nakahira 2005), which is common to *L. equestris* (Solbreck and Sillén-Tullberg 1990; Tullberg *et al.* 2000).

As found here, population variation in longevity, fecundity, egg size, adult size/mass, was also found in a previous study of two *L. equestris* populations (Shuker *et al.* 2006), suggesting that such differences may be common. Additionally, development time variation was as great within *L. equestris* as it was between the two species here, and *L. simulans* although producing the smallest eggs, were heavier as adults. Thus, the populations and species clearly differ in many aspects of their life-history, and the variation is often as great within *L. equestris* populations as it is between the two species in the laboratory. This suggests that populations may differ in aspects of energy allocation to reproduction. However, although only 6 populations were tested here (and in two sets of three) no consistent patterns between female mating costs and life history differences emerge (data not shown). Few studies have directly studied the extent of sexual conflict over multiple field populations to asses its potential variation, however, in the water strider *Aquarius remigis*, population structure has a strong influence on sexual conflict (Eldakar *et al.* 2009a; Eldakar *et al.* 2010a). Male manipulation of females (aggression and harassment) positively correlates with mating success, causing reduced female fitness and consequently the fitness of the group when isolated, in a tragedy of the commons type situation (Eldakar *et al.* 2009a; Eldakar *et al.* 2009b). However, allowing dispersal among groups resulted in aggressive males being able to avoid the costs of reduced population fitness, and led to variation in sex ratios, and degree of harassment, across populations and consequently lower levels of conflict overall (Eldakar *et al.* 2010b). Indeed, in the wild dispersing males have been shown to be significantly more aggressive than non-dispersing males (Eldakar *et al.* 2010a), and dispersal of females away from local aggression created more favourable environments for less aggressive males in the lab (Eldakar *et al.* 2010b), as is apparent in ephemeral streams in the wild (Eldakar *et al.* 2010a). Similarly, habitat use is important to the sexual conflict over

mating in the guppy *Poecilia reticulate*, with females using faster moving water to avoid excessive male mating attempts (Magellan and Magurran 2006; Darden and Croft 2008). Thus behavioural avoidance of males to restrict detrimental effects of sexual conflict to females may be common in the field. Further study of more populations is needed to elucidate general patterns and associations between sexual conflict and life-history evolution in the field.

The substantial fitness costs of high mating treatments found here demonstrates that sexual conflict over mating may be considerable in the field. Population variation in many life history characters was also apparent, most likely shaped by local ecology. The extent to which selection on males and females over mating (e.g. male persistence and female resistance) either directly influences life-history (by contributing to the cost of reproduction for instance), or is instead influenced by life-history and/or ecology (e.g. from energy available for reproduction through to operational sex ratio) remains unclear. I found no patterns (albeit with a limited number of populations) to suggest an association between life-history traits and sexual conflict over mating. However, hopefully this work will provide useful data for both ongoing work on this system and for future comparative studies. Identifying sexual conflict in field populations can allow predictions of theoretical consequences of sexual conflict to be tested. Specifically, sexual conflict theory has proposed that a number of possible evolutionary outcomes may result from conflict, ranging from restricting, to promoting, population divergence and speciation (Parker and Partridge 1998; Gavrilets and Waxman 2002; Gavrilets and Hayashi 2005). Indeed, the next stage of our research is to investigate the mating behaviour of these populations in inter-population crosses to elucidate if the apparent sexual conflict over mating in these populations is associated with SAC driving the populations along divergent evolutionary trajectories (Parker 1979; Holland and Rice 1998; Parker and Partridge 1998; Martin and Hosken 2003). For example, SAC could potentially prevent hybridisation if specific signal-receptor systems differ among the populations due to differences among populations in their respective male persistence and female resistance to mating mechanisms.

Chapter 5

Reproductive isolation within and between species characterised by a sexual conflict over mating

Abstract

Theory suggests that under some circumstances sexual conflict over mating can lead to divergent sexually antagonistic coevolution (SAC) among populations facilitating reproductive isolation. However, its relative importance in the wild has been difficult to ascertain, and the generality of conflict promoting divergence has been questioned. To test this theory one needs populations evolving under sexual conflict, and to subsequently test for divergence in reproductive characters. Here I compare pre- and post-mating isolation within and between species to explore how populations and species diverge in the face of sexual conflict. I aim to quantify sexual isolation among five populations of *Lygaeus equestris*, and two replicated populations of *Lygaeus simulans*, that are characterised by sexual conflict over mating. I find no evidence of reproductive isolation amongst populations of *L. equestris*, and thus no evidence that sexual conflict associates with population divergence in relevant mating traits in *L. equestris*. However, there was strong pre-mating isolation between *L. equestris* and *L. simulans* and this was asymmetric in form; male *L. simulans* were able to mate with female *L. equestris*, but male *L. equestris* were largely unable to mate with female *L. simulans*. I found little evidence for strong post-mating isolation between the two species however, with hybrid F_2 offspring being produced. The results confirm that sexual conflict need not lead to population divergence that results in sexual isolation, and indeed perhaps support the contrary theoretical proposition that male willingness to mate may retard speciation through promoting gene flow.

Introduction

Sexual conflict, where sexually divergent optima exist for a given trait, is expected to be ubiquitous among sexually reproducing species (Parker 1979; Arnqvist and Rowe 2005). It has been proposed as an important driver of evolutionary change, particularly if it results in sexually antagonistic co-evolution (SAC). Within populations, SAC from inter-locus sexual conflict may be rapid, resembling irresolvable races between the sexes, or cyclical dynamics as males and females constantly co-evolve in response to adaptations and manipulation of the other sex (Parker 1979; Rice 2000; Arnqvist and Rowe 2005; Lessells 2006). Furthermore, SAC has received much attention due to its potential to drive populations along divergent co-evolutionary trajectories, facilitating population divergence, sexual isolation, and indeed speciation (Holland and Rice 1998; Parker and Partridge 1998; Gavrilets 2000; Martin and Hosken 2003; Arnqvist and Rowe 2005).

Males are typically thought to be selected to mate more often than is optimal for females (Bateman 1948; Trivers 1972), leading to sexual conflict over mating. Laboratory studies have convincingly demonstrated that sexual conflict over mating can lead to SAC, with the evolution of increased (decreased) female resistance to mating under situations of increased (decreased) conflict over mating (Holland and Rice 1999; Wigby and Chapman 2004; Stewart *et al.* 2005; Rice *et al.* 2006). Such SAC over mating rate can result in population divergence (Hosken *et al.* 2002; Martin and Hosken 2003), but divergence has not always resulted (Wigby and Chapman 2006; Bacigalupe *et al.* 2007; Gay *et al.* 2009; Maklakov *et al.* 2010). However, SAC may be less common in the field (Chapman 2006), and studies conclusively showing the past operation of SAC remain limited in number, (Arnqvist and Rowe 2002a; Arnqvist and Rowe 2002b; Koene and Schulenburg 2005; Bergsten and Miller 2007; Anthes *et al.* 2008). Fewer studies still, demonstrate active SAC within species to be driving current population divergence in the field (e.g. Gagnon and Turgeon 2011; but see Hebets and Maddison 2005; Sugano and Akimoto 2007).

Thus, although sexual conflict is apparent in nature, its importance for evolutionary divergence has been questioned. Recent theory shows the consequences of SAC for evolution are difficult to interpret (e.g. Gavrilets and Hayashi 2005), and conflict is sensitive to environmental stochasticity. Indeed, evidence of context dependent expression of sexual conflict over mating is mounting. For example, conflict over mating may be affected by ecological parameters, such as habitat and population structure, that influence encounter

rates as found in seaweed flies (Edward and Gilburn 2007), water striders (Eldakar *et al.* 2009a; Eldakar *et al.* 2010a), and guppies (Magellan and Magurran 2006, see also Härdling and Kaitala 2005; Kokko and Rankin 2006). Additionally, food availability is known to mediate the costs of mating in fruit flies (Chapman and Partridge 1996) and moths (Wedell *et al.* 2002b), whilst predation pressure can also have strong implications for the extent of conflict experienced (Magnhagen 1991; Arnqvist 1997; Lode *et al.* 2004; Arnqvist and Rowe 2005; Elgee *et al.* 2010). These examples indicate that the general expression of conflict may be substantially lower than expected from first principles. Indeed more recent theoretical studies suggest that population divergence from sexual conflict may be much less likely than first thought, even when SAC is apparent, with only two of six possible SAC dynamics resulting in population divergence (Gavrilets and Hayashi 2005). This may account for the lack of population divergence observed amongst some laboratory evolution studies (e.g. Wigby and Chapman 2006; Bacigalupe *et al.* 2007; Maklakov *et al.* 2010)

Measuring the degree of sexual isolation among allopatric populations that show variation in sexual conflict over mating is one way to gain insight into the role of sexual conflict in population divergence and speciation in the field (Gay *et al.* 2009; Gagnon and Turgeon 2011). The utility of studying inter-population crosses, in common garden laboratory experiments, to examine the extent of sexual conflict over mating has been questioned (Long *et al.* 2006), as SAC may not be expected to produce any particular pattern in inter-population mating crosses (Chapman *et al.* 2003; Long *et al.* 2006; Tregenza *et al.* 2006). However, examining sexual isolation among populations known to differ in sexual conflict can be valuable in reporting the outcome of conflict for evolution in the field. Indeed, population crosses can be important as a first step towards identifying current episodes of population divergence, and in exploring the involvement of sexual conflict and SAC in such diversification episodes (e.g. Hebets and Maddison 2005; Long *et al.* 2006; Panhuis *et al.* 2006; Sugano and Akimoto 2007).

As mentioned above, reproductive isolation and population divergence may be promoted under conditions of sexual conflict (Holland and Rice 1998; Parker and Partridge 1998; Martin and Hosken 2003). However, SAC over mating is expected to select for generally persistent, manipulative, males and female resistance to male mating attempts (Parker and Partridge 1998). Thus, heterotypic mating may occur more readily than conspecific mating, if females are less able to resist foreign males (that they have not co-evolved with, Jennions and Petrie 1997). Therefore, sexual conflict may actually retard

population divergence, rather than promote it, by maintaining gene-flow across populations (Holland and Rice 1998; Markow and Hocutt 1998; Parker and Partridge 1998; Gavrilets *et al.* 2001).

Alternatively, under sexual conflict a certain 'threshold' of male persistence for mating may be required to induce females to mate (Gavrilets *et al.* 2001). Different populations (that may experience differing levels of sexual conflict) could therefore differ in these threshold values, resulting in asymmetric mating among populations (Markow and Hocutt 1998; Pizzari and Snook 2003; Sugano and Akimoto 2007). For instance, males with high persistence levels may readily mate foreign females (with lower resistance thresholds), whilst the reciprocal cross (low persistence males with high resistant females) may not result in mating (Markow and Hocutt 1998; Pizzari and Snook 2003; Sugano and Akimoto 2007). This would also slow, and possibly prevent, population divergence by maintaining gene-flow, albeit asymmetrically across populations. If, on the other hand, female receptivity to males is plastic, and female indifference, or sensitivity, to male persistence evolves (Rosenthal and Servedio 1999; Rowe *et al.* 2005), the consequences for inter-population breeding become more complicated, and it is unclear whether population divergence would be promoted or hindered (Rowe *et al.* 2005). In speciation, pre-zygotic sexual isolation is expected to evolve faster than post-zygotic sexual isolation, particularly in sympatry, due to the reinforcement by natural, and sexual, selection against hybrids of reduced fitness (Coyne and Orr 1989; Coyne and Orr 1997). Furthermore, populations with little pre-zygotic sexual isolation are likely to fuse, or lead to the extinction of hybrids, thus reducing instances of post-zygotic reproductive isolation (Coyne and Orr 1989; Andersson 1994; Coyne and Orr 1997).

Here I explore patterns of pre- and post-mating reproductive isolation among multiple populations of two closely related species of seed bugs; *Lygaeus equestris* (L) and *Lygaeus simulans* (Deckert 1985). These species have similar ecologies, including aposematic warning colouration, and both show promiscuous mating systems characterised by sexual conflict (Deckert 1985; Tadler 1999; Tadler *et al.* 1999; Micholitsch *et al.* 2000; Shuker *et al.* 2006). Two *L. equestris* populations were derived from Sweden, three from Northern Italy, and two *L. simulans* populations were derived from Central Italy. Firstly, I predicted that *L. simulans* and *L. equestris* are 'good' species and that they would be both pre- and post-zygotically isolated, confirming (albeit limited) observations thus far made concerning the lack of hybrids in the wild (Maschler 2002). Given that the two species are

presumed sister species, pre-zygotic isolation is expected to be more advanced than the post-zygotic isolation (Coyne and Orr 2004). Secondly, if sexual conflict is associated with heightened population divergence through divergent SAC, then one should expect variation in the extent to which populations of *L. equestris* mate with each other, and perhaps also in their hybrid offspring viability. On the other hand, if the populations have diverged in mating-related traits, and become isolated due to ecological factors, then we might expect the Swedish and Italian populations to be more likely to mate with individuals from the same region. I performed two sets of within- and between-species no-choice mating experiments to test these predictions. The first (in collaboration with Toby Nowlan) assayed mating propensity over a short period for reproductively mature individuals, allowing investigation of the latency of individuals to mate among four populations. The second, larger, experiment expanded the number of populations studied in the first experiment to seven populations, and assayed mating over a prolonged period of adult life, as well as the production of an F_1 and an F_2 offspring generation.

Methods

The study species

In total, seven populations of *Lygaeus* (Hemiptera: Lygaeidae) were used in the experiments described below, including four populations of *L. equestris* (L) and two populations of its sister species, *L. simulans* (Deckert 1985, see Appendix 1 for morphological and species identification). *Lygaeus simulans* differs to *L. equestris* in the morphology of the antennae base, and male parameres (genital claspers, Deckert 1985; Péricart 1998). Much of the ecology of the two species is also similar (see Chapter 2 for details).

General methods

To ensure a continual supply of bugs for the experiments, I transferred late larval instars periodically (every 4-5 days) from culture cages into smaller nymph development cages for adult eclosion (see Chapter 1 for general husbandry of bugs). Two experiments are reported here; a four population experiment and a seven population experiment. In the four population experiment, mating was examined for sexually mature individuals, in a 4*4 population reciprocal cross design (i.e. 16 replicated combinations created, see Appendix 2, Figure A) from February to April 2010. The populations included two Swedish *L. equestris* populations (Morga and Geta, sampled in 2008), and a laboratory adapted, *L. equestris* population from the Dolomites region of northern Italy (sampled in 2004 by Dr David Shuker). The fourth population was *L. simulans* sampled from the Tuscany region of central Italy (Tuscany population) in 2008 by Dr David Shuker. Here, both mating propensities (i.e. the incidence of mating), and time to mating, where apparent, were explored across the populations and species. The seven population experiment, performed in August to December 2010, extended the four population experiment, with modifications. These included two further *L. equestris* populations from Northern Italy (Ledro and Predazzo), and another *L. simulans* population from Tuscany (Tuscany.2, all sampled in 2009, see Chapter 2 for further details concerning these populations, and Appendix 1 for morphological comparisons). Thus, a 7*7 population reciprocal cross mating design was performed (i.e. 49 replicated combinations, see Appendix 2, Figures B and C). Here, I explored mating over a prolonged period of adult development (from adult eclosion). Additionally, further aspects of pre- and post-mating sexual isolation

were investigated. I reared any F_1 generation offspring produced, and allowed them to reproduce, thus enabling assessment of hybrid F_1 fertility.

Four population experiment

I removed newly eclosed adults from nymph cages every two days, and placed them in same sex pots (within their respective populations) with others of the same age to develop, ensuring virginity. Densities were restricted to six bugs per pot (measuring 8 x 8 x 5.5cm, transparent with perforated lids). Each pot also contained small (30mm diameter) Petri dish lids and bases containing water soaked cotton wool and organic, de-husked, sunflower (*Helianthus*) seeds (Goodness Direct, Northampton). Seeds and water were replaced every three days ensuring an *ad-libitum* supply of both throughout the experiment. All bugs were retained in these conditions for at least seven days prior to experimentation ensuring sexual maturity upon experimentation (see Chapters 2 and 3).

Adult bugs aged between seven and 12 days post adult eclosion, were used in 'no-choice' mating treatments. I randomly assigned sexually mature males and females to partners of the opposite sex from each of the four populations (Morga, Geta, Dolomites *L. equestris*, and Tuscany *L. simulans*) in a fully-factorial reciprocal cross mating design (Appendix 2, Figure A). Pairs were placed in transparent pots, without seeds or water, and were randomly distributed in trays, before returning to an incubator (29°C, 22:2 L:D duration). I scored the pairs every 30 minutes, for eight hours, for copulation (stable end to end position Sillén-Tullberg 1981; Tadler *et al.* 1999). Each hour, pots were rotated in their position within the incubator to minimise potential position effects. Pairing treatments were performed in blocks, with at least two replicates of each combination attained per block. Pairs where one or both individuals died during the observational period were discarded, and not included in the analysis. From the eight hour observation period, I calculated whether mating occurred and, for each pair that mated, the time taken for mating to occur. Ultimately 25 replicates were gained for each reciprocal cross (total $N = 400$ pairings).

Seven population experiment

I repeated the experiment above, but males and females from seven populations were paired in a reciprocal cross–population mating design yielding 49 pair combinations (including within population pairings). For this experiment, males and females were paired from 0-2

days post adult eclosion and retained in small pots (8 x 8 x 5.5cm) containing a layer of organic, de-husked, sunflower seeds, and a 7ml bijou tube (Fisher scientific) containing carbon filtered water capped with a cotton wool bung, for a maximum of 20 days. Water tubes were replaced every 10 days, or when necessary, to ensure a constant supply of water. From days 3 to 12 of being paired (bugs aged ~ 5 to 14 days post eclosion, allowing for the necessary development time required for sexual maturity, see Chapters 2 and 3), I scored pairs twice daily for mating. Males that died during this time, without mating being observed, were replaced by another virgin male of similar age from the appropriate population. Where males died and mating was observed the female was left in isolation to oviposit. Males were transferred to a new female if a female had died during this time with no mating observed, and no eggs oviposited. However if a female died after producing eggs, the eggs (within the pot) were retained and left to develop, with the male discarded. After day 12, adults were left to continue mating (without scanning for mating) and females left to oviposit eggs until day 20, whereupon both adults were removed and discarded.

Following removal of the adults on day 20, eggs were left to develop in their respective pots without their parents. After a further 7 days (when all viable eggs have hatched), I scored pots for the presence of offspring (hatched eggs), and their numbers recorded. I then transferred up to 25 hatchlings to a fresh pot with seeds and water tube (as above) to develop with the rest discarded. Larger (older) nymphs were preferentially retained to shorten the experimental duration. Pots were then checked for adult eclosion every 5 days and a maximum density of 5 males and 5 females were retained together in fresh pots upon eclosion. Where either no males or no females eclosed (i.e. one sex absent), virgin bugs from other replicated pots (of the appropriate cross) were used and transferred to the remaining bugs where possible to allow for mating and reproduction of this F_1 generation to be assayed. Once again, water was replaced every 10 days, or earlier, to allow for a constant supply. I then retained all pots for up to 20 days and scored them for the presence/absence of F_2 offspring (hatched eggs) to determine F_1 generation fertility.

As with the four population experiment, this experiment was ‘rolling’ in form, where pairs were set up, in blocks, as bugs became available. Ultimately, between 8 and 14 replicate pairs per combination (reciprocal crosses) were gained to observe mating interactions (median N = 10, see Appendix 2, Figures B and C). Out of the 491 replicates obtained, 12 replicates produced an F_1 generation without mating observed. Mating events may have been missed in these crosses, but more likely these pairs mated after the 9 day

observation period (particularly if one or other of the partners died and replaced). All combination crosses, that produced F_1 offspring, showed mating to occur between at least a subset of the replicate pairs however, indicating that twice daily observational scans were adequate for assessing mating (as is likely given the prolonged copulation durations exhibited within these species). I used a threshold of at least 3 F_1 adult males and 3 F_1 adult females to test for the production of F_2 offspring, unless F_2 offspring were produced from fewer adults. Of the 223 replicates that successfully produced F_1 offspring, 22 had insufficient numbers of adults emerging (due to death or too few of one or both sexes) to reliably assay for the production of F_2 offspring within the respective replicate families.

Statistical analysis

Four population experiment

Mating and sexual isolation

I analysed mating propensity, in terms of the incidence of mating, using binary logistic regression with a logit link function (Crawley 2007). Firstly, I analysed mating within populations only, to examine if there were population differences in baseline mating propensities. Secondly, male and female identities (their population origin) were used as factors, along with the interaction term between them to test for differences in likelihood of mating across all population crosses. Finally, I performed an intraspecific analysis of *L. equestris* populations to investigate potential differences in mating propensity across *L. equestris* populations. Sexual isolation is indicated by significant interaction effects, and was assessed in each case using likelihood ratio (LR) tests.

I further analysed sexual isolation among the populations and species using an overall sexual isolation index (*I*PSI, Rolan-Alvarez and Caballero 2000; Perez-Figueroa *et al.* 2005). This was performed as a global analysis ($I_{PSItotal}$), as well as for each pairwise comparison ($I_{PSIa,b}$), and estimates of asymmetry among these crosses ($I_{APSIa,b}$) were also tested (Carvajal-Rodriguez and Rolan-Alvarez 2006). I_{PSI} values range from -1 to 1, where 0 = random mating, -1 = complete dis-assortitive mating, and +1 = complete assortative mating (sexual isolation). I_{APSI}, values range from 0 to infinity, were asymmetry in mating is indicated by I_{APSI} values significantly smaller or greater than 1 (e.g. Schwartz and McPheron

2007; Jennings and Etges 2010). Significance of these sexual isolation indices was tested using bootstrap resampling with 10,000 iterations. Where no mating was observed among pairs, zeros were replaced with 0.5 to allow for bootstrap resampling. Tests of sexual isolation were performed using the programme JMATING (Carvajal-Rodriguez and Rolan-Alvarez 2006).

Time to mating

I analysed reproductive interactions among mating pairs, in terms of the time taken to mate (assayed by scanning for mating every 30 minutes), using a GLM with a quasi-poisson error distribution and log link to account for overdispersion of the data (Crawley 2007). As with mating propensity above, I analysed time to mating, firstly within populations only, secondly, for all crosses, and subsequently, for *L. equestris* populations only.

Seven population experiment

F_1 Progeny production

Mating propensity and sexual isolation among the populations and species was assessed in the same manner as performed for the four population experiment above. Here, however, mating success (the incidence of F_1 progeny production from those that did mate), and the number of progeny produced (of those that produced offspring) from each mating pair was also tested. I analysed the incidence of F_1 progeny production among pairs with logistic regression, firstly for conspecific pairs (within populations), and subsequently using all crosses to examine the effects of male and female identity and their interaction on mating success respectively. This was then repeated using only the subset of pairs that mated to investigate potential post mating reproductive barriers, firstly for all pairs and, secondly, among *L. equestris* populations only.

I performed ANOVAs on the number of progeny produced (by pairs that produced offspring) again firstly for conspecific pairs (within populations), and subsequently using all crosses to examine effects of male and female identity and their interaction on fecundity respectively (factorial ANOVA). Lastly, *L. equestris* populations were further analysed to test for differential mating success in terms of the number of hatched offspring produced by reproductive pairs. Where the interaction term was not significant, it was removed and the

model refitted using only the main effects. These analyses were performed using R (R version 2.11.1).

Results

Four population experiment

Mating and sexual isolation

Mating propensity within each population was similar across all the populations, and species (mean proportion mating = 0.89, N = 100). There were no differences among the populations, or species, in mating propensity when paired with conspecific (local) partners (LR χ^2 = 3.00, df = 3, p = 0.392, Figure 1). The main differences arose from the inter-specific pairings of *L. equestris* and *L. simulans* (Figure 1), where the reduced mating among inter-specific pairs showed substantial gender asymmetry. Thus, the analysis, including all the reciprocal population crosses, revealed a highly significant interaction effect between male and female identity (i.e. the population derived from) on the incidence of mating (N = 400, interaction effect; χ^2 = 231.12, df = 9, p < 0.0001, main effects; male population χ^2 = 40.55, df = 3, p < 0.0001, female population χ^2 = 71.22, df = 3, p < 0.0001). Within *L. equestris*, Morga males mated less overall than males from other populations (N = 225, χ^2 = 9.167, df = 2, p = 0.01, Figure 1), but for females there were no population differences in the incidence of mating (χ^2 = 0.348, df = 2, p = 0.840) and no interaction between male identity and female identity on the incidence of mating (χ^2 = 4.73, df = 4, p = 0.316).

Specifically looking at the nature of the heterogeneity in mating between the populations I found strong pre-mating sexual isolation between each of the *L. equestris* populations (Morga, Geta, and Dolomites) and *L. simulans* respectively (Total I_{psi} = 0.573, p < 0.001, Table 1), but no sexual isolation effects among *L. equestris* populations (see Table 1). However, the sexual isolation effect between *L. equestris* and *L. simulans* was asymmetric (I_{Apsi} = 4.065, p = 0.005) with *L. simulans* males able to mate with *L. equestris* females whilst the opposite was not found (Figure 1, Table 1).

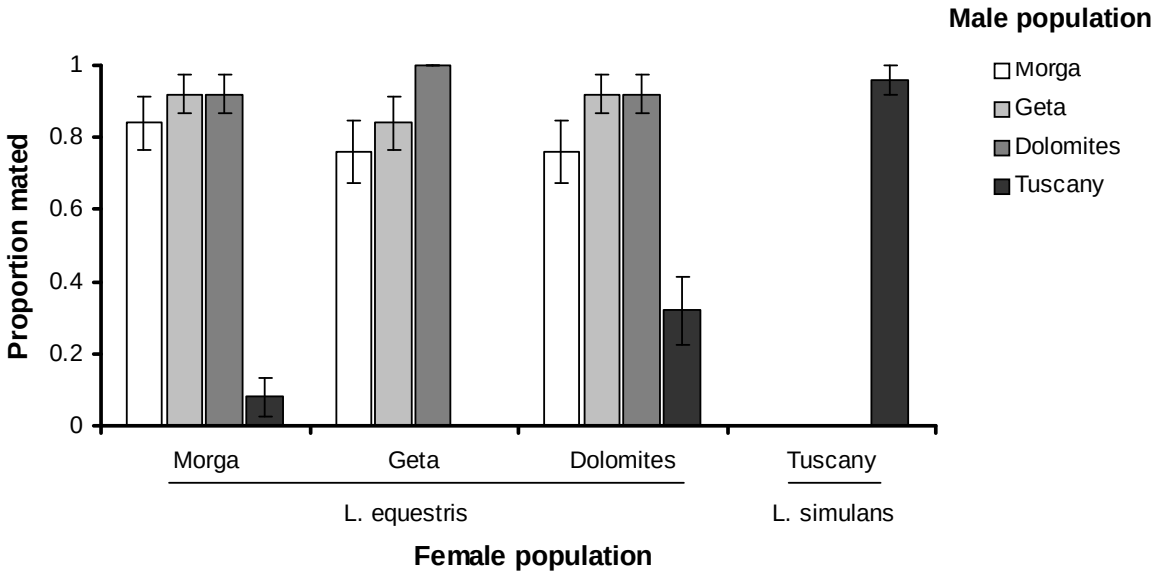

Figure 1. Proportion of pairs mating for each combination within and between populations and species (N = 25 each, total N = 400) in the four population experiment. Morga, Geta, and Dolomites populations are *Lygaeus equestris*. The Tuscany population is the sister species, *L. simulans*. Error bars are standard errors for proportions. See text for further details.

Time to mating

Overall, there was little difference within, or between, species in time to mating, where mating occurred. The time to mating did not differ among the populations and species when paired with conspecific partners (mean = 1.43 hours, se = 0.15, $F_{3,85}$ = 0.157, p = 0.924, Figure 2). Additionally, including heterospecific pairings showed no interaction effect between male and female identity on the time to mating ($F_{5,219}$ = 1.417, p = 0.219), and no overall difference among any of the populations or species in the time taken for males or females to mate (male population; $F_{3,224}$ = 1.482, p = 0.220, female population; $F_{3,224}$ = 0.248, p = 0.863, Fig. 2). Analysing only *L. equestris* populations gave the same qualitative results of no difference in time to mating for any of the factors (data not shown, see Figure 2.).

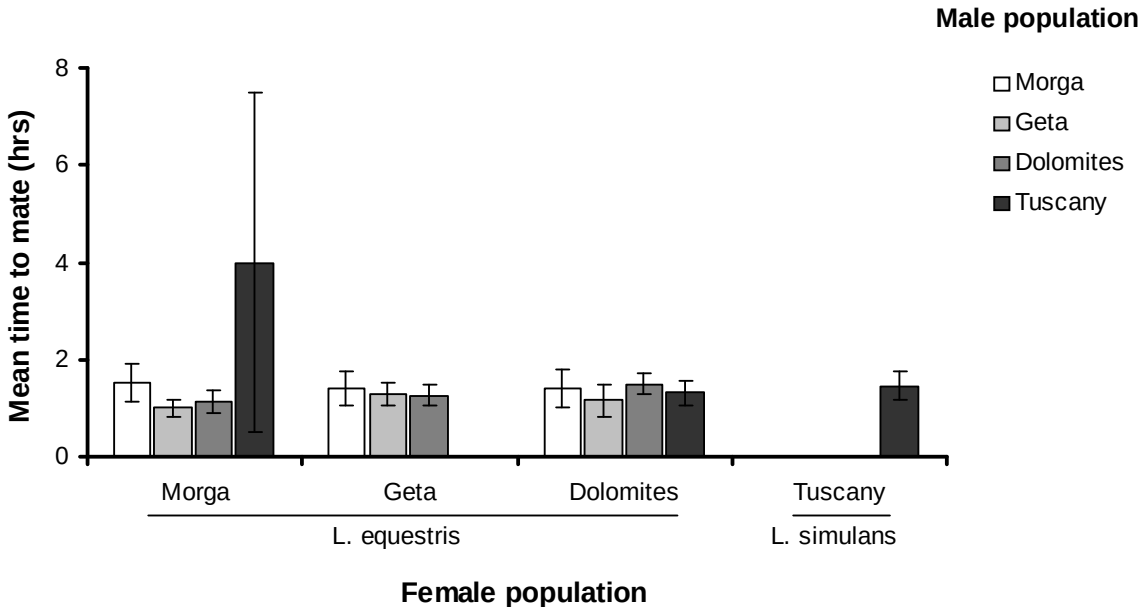

Figure 2. Mean time to mating for pairs that did mate within and between populations, and species, in the four population experiment. Error bars are standard errors. The Morga, Geta and Dolomites populations are *Lygaeus equestris*. The Tuscany population is the sister species, *L. simulans*.

Seven population experiment

Mating and sexual isolation

As with the four population experiment, there was no difference between the populations, or species, in their mating propensities when paired with conspecific partners (mean incidence = 0.813, $N = 64$, LR $\chi^2 = 7.32$, $d.f. = 6$, $p = 0.29$). However, once again, mating between the species was much lower than within them, and was asymmetric in form. Including hetero-specific pairings revealed significant heterogeneity among the populations in the likelihood of mating, as expected for different species ($N = 491$, interaction effect: LR $\chi^2 = 225.11$, $d.f. = 36$, $p << 0.0001$, main effects male population; LR $\chi^2 = 1.85$, $d.f. = 6$, $p = 0.93$, female population; LR $\chi^2 = 126.44$, $d.f. = 6$, $p << 0.0001$, Figure 1). Within *L. equestris* populations, the mating propensity of males did not differ with the identity of the female partner, and vice versa ($N = 255$, interaction effect; LR $\chi^2 = 22.94$, $d.f. = 16$, $p = 0.134$), and no population differences were found between the overall mating propensity of males (LR $\chi^2 = 3.14$, $d.f. = 4$, $p = 0.536$). However the populations did differ in the overall mating propensity of females (LR $\chi^2 = 11.175$, $d.f. = 4$, $p = 0.025$), suggesting that the populations differ in baseline female

receptivity to mating (Figure 3). Again, closer examination of reproductive isolation amongst the experimental populations using *Ipsi* statistics reveal significant pre-mating sexual isolation between the two species, but not within species (Total *Ipsi* = 0.255, p = 0.006; Table 1). Moreover, as with the four population experiment, sexual isolation between the species was significantly asymmetric when *L. equestris* from Ledro, Morga, and Predazzo, were paired with *L. simulans* respectively (Table 1, Figure 3).

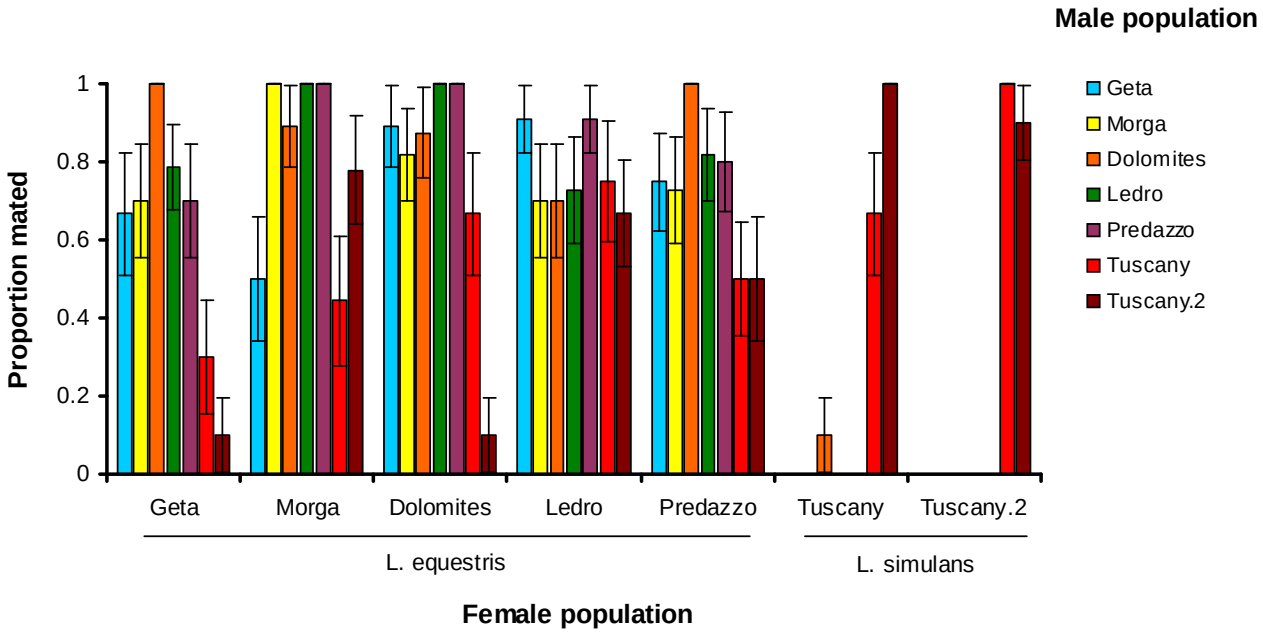

Figure 3. Seven population experiment. Proportion of females mating when paired with a male of the same or different, population and species, respectively. Error bars are standard errors for proportions. See text for further details.

Table 1. Global analysis of sexual isolation (*Ipsi*), and estimates of asymmetry (*IApsi*) in mating among populations using F_0 crosses for the four and seven population experiments respectively. SD is the standard deviation and *p* is the two tail probability of rejecting the null hypothesis being true (isolation/asymmetry = 0) in the bootstrap resampling distribution (derived from 10,000 iterations). Crosses displaying significant pre-mating isolation are shown in bold. See text for details.

Population Cross	***Ipsi***	**SD**	***p***	***IApsi***	**SD**	***p***
Experiment 1						
Morga * Geta	0.001	0.110	0.9922	1.003	0.044	0.935
Morga * Dolomites	0.024	0.109	0.8368	1.009	0.049	0.972
Morga * Tuscany	0.902	0.060	< **0.0001**	1.505	1.006	0.535
Geta * Dolomites	-0.044	0.107	0.6642	0.998	0.034	0.869
Geta * Tuscany	0.958	0.042	< **0.0001**	0.438	0.620	0.543
Dolomites * Tuscany	0.753	0.070	< **0.0001**	4.065	1.235	**0.005**
Total	0.573	0.049	< **0.0001**			
Experiment 2						
Dolomites * Geta	-0.137	0.197	0.456	1.019	0.121	0.977
Dolomites * Ledro	-0.030	0.195	0.857	1.013	0.164	0.834
Dolomites * Morga	-0.009	0.185	0.948	1.011	0.131	0.888
Dolomites * Predazzo	-0.120	0.183	0.492	0.996	0.116	0.795
Dolomites * Tuscany	0.440	0.217	0.079	1.621	0.525	0.146
Dolomites * Tuscany.2	0.844	0.131	**0.001**	0.739	0.658	0.947
Geta * Ledro	-0.208	0.177	0.235	0.990	0.120	0.775
Geta * Morga	0.140	0.209	0.513	1.091	0.293	0.892
Geta * Predazzo	-0.069	0.199	0.702	1.014	0.132	0.993
Geta * Tuscany	0.640	0.192	**0.021**	1.423	0.533	0.268
Geta * Tuscany.2	0.835	0.142	**0.005**	0.725	0.642	0.941
Ledro * Morga	0.011	0.178	0.964	0.988	0.138	0.895
Ledro * Predazzo	-0.086	0.180	0.600	1.001	0.107	0.840
Ledro * Tuscany	0.539	0.176	**0.022**	1.851	0.523	**0.045**
Ledro * Tuscany.2	0.541	0.144	**0.008**	2.202	0.619	**0.016**
Morga * Predazzo	-0.002	0.179	0.974	1.016	0.142	0.906
Morga * Tuscany	0.647	0.162	**0.010**	1.763	0.584	0.123
Morga * Tuscany.2	0.589	0.134	**0.001**	2.354	0.642	**0.013**
Predazzo * Tuscany	0.539	0.176	**0.027**	1.853	0.533	**0.047**
Predazzo * Tuscany.2	0.627	0.145	**0.004**	2.016	0.611	0.056
Tuscany * Tuscany.2	-0.101	0.187	0.560	0.994	0.134	0.756
Total	0.255	0.081	**0.005**			

F_1 offspring production

Fecundity, in terms of the incidence of hatched offspring production, did not differ between populations when paired with conspecific mates as expected (mean = 0.641, $N = 64$, LR $\chi^2 = 5.824$, $d.f. = 6,57$, $p = 0.44$). However, including all pairs, there was strong heterogeneity amongst the populations in the likelihood of interbreeding, revealed by a significant interaction effect between male and female identity on the incidence of hatched offspring production ($N = 491$, interaction effect; LR $\chi^2 = 167.74$, $d.f. = 36$, $p << 0.0001$, male population; LR $\chi^2 = 16.32$, $d.f. = 6$, $p = 0.012$, female population; LR $\chi^2 = 94.30$, $d.f. = 6$, $p << 0.001$, Figure 4). This was largely due to the effects of pre-mating isolation shown above. Indeed, reducing the data set to contain only those pairs that did mate, revealed that the likelihood that a male from a particular population produced hatched offspring with a female, did not depend on the identity of the female partner, and vice versa ($N = 305$, interaction effect; LR $\chi^2 = 36.46$, $d.f. = 27$, $p = 0.106$), suggesting little post-mating reproductive isolation between the species. However, there were still population differences, for both males and females, in the likelihood of producing hatched offspring following successful mating however (male population; LR $\chi^2 = 34.35$, $d.f. = 6$, $p < 0.001$, female population; LR $\chi^2 = 25.99$, $d.f. = 6$, $p < 0.001$). Contrary to the result of no population differences in the likelihood of producing hatched offspring in conspecific pairings, this suggests that there is some variability among the populations in the likelihood of producing hatched offspring (Figure 4). Indeed, analysis of *L. equestris* populations only, showed a similar story to mating propensity, with a significant effect of female population identity on the incidence of hatched offspring among mated pairs, but no effect of male identity and no interaction between the two ($N = 218$, LR: female identity $\chi^2 = 17.128$, $d.f. = 4$, $p = 0.002$; male identity $\chi^2 = 6.022$, $d.f. = 4$, $p = 0.198$; interaction term $\chi^2 = 17.966$, $d.f. = 16$, $p = 0.326$).

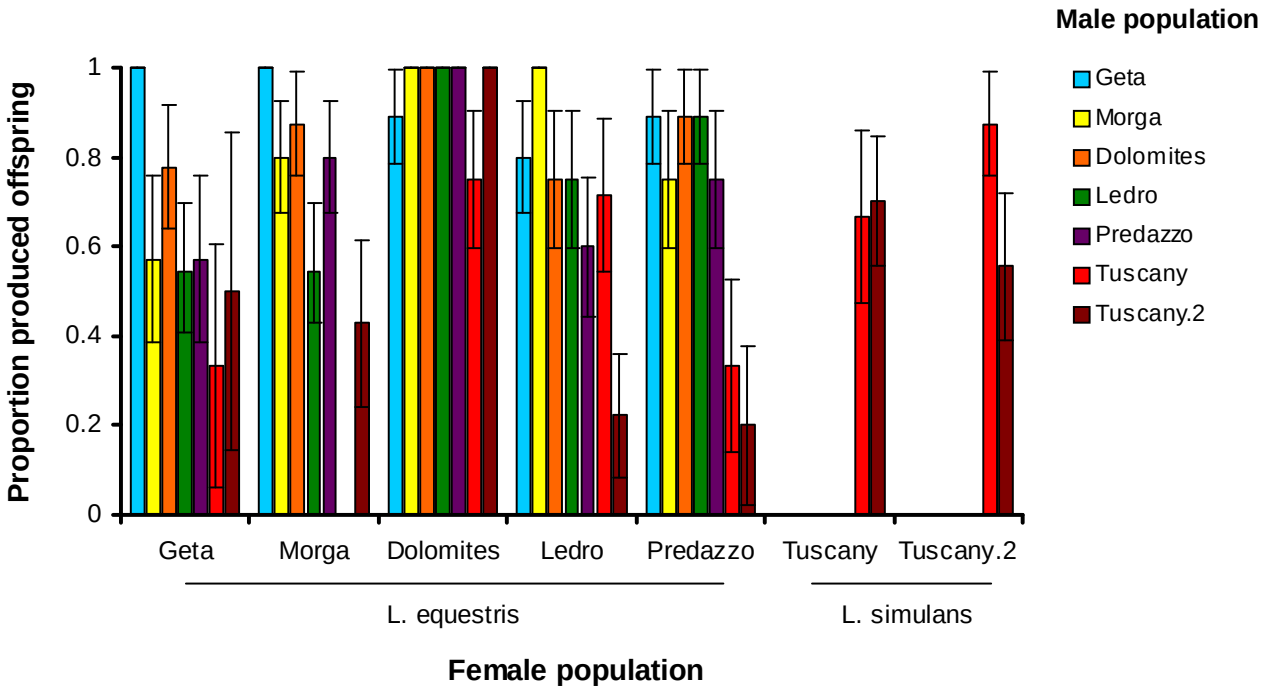

Figure 4. Seven population experiment. Proportion of mated females producing hatched offspring when paired with a male of the same or different, population and species, respectively. Error bars are standard errors for proportions. See text for further details.

There was no difference among the populations in the number of hatched offspring produced (by reproductive pairs) when paired with conspecific partners (mean = 59.88, se = 5.67, $F_{6,35}$ = 1.067, p = 0.401). Analysing all population crosses together, I found that the number of offspring produced by females did not depend on the identity of the male partner (interaction effect; $F_{25,183}$ = 0.85, p = 0.674), suggesting there to be little difference in the numbers of offspring produced between hybrid crosses and within species. However this is likely to be due to a lack of power to detect a difference resulting from the low sample sizes (see Figure 5). There were highly significant population differences for males and females in the number of hatched offspring produced overall (male population; $F_{6,183}$ = 9.29, p << 0.0001, female population; $F_{6,183}$ = 7.49, p << 0.0001), which appears to be driven largely by the low numbers of offspring produced among *L. equestris* and *L. simulans* population crosses (although as noted above the interaction was not found to be significant, Figure 5, see also Appendix Figure C). Analysing *L. equestris* populations in isolation, again there was no significant interaction effect between male and female identity on the number

of offspring produced ($F_{16,151}$ = 1.167, p = 0.301), however, male and female identity were significant as main effects (male $F_{4,167}$ = 3.117, p = 0.017, female $F_{4,167}$ = 2.731, p = 0.031), suggesting there is variance in fecundity across populations.

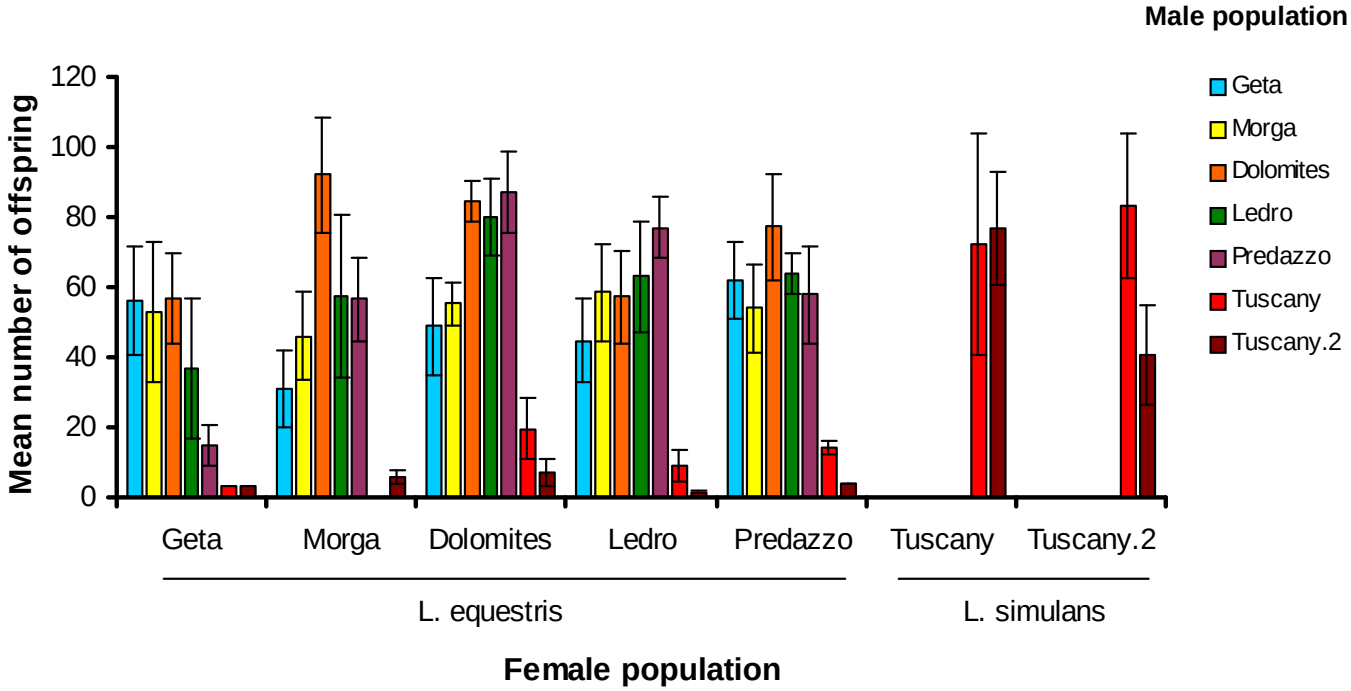

Figure 5. Seven population experiment. Mean number of hatched F_1 offspring produced for reciprocal cross pairs that did reproduce within and between populations and species respectively. Error bars are standard errors.

			Female						
			L. equestris					L. simulans	
			Geta	Morga	Dolomites	Ledro	Predazzo	Tuscany	Tuscany.2
Male	L. equestris	Geta	✓	✓	✓	✓	✓	NA	NA
		Morga	✓	✓	✓	✓	✓	NA	NA
		Dolomites	✓	✓	✓	✓	✓	NA	NA
		Ledro	✓	✓	✓	✓	✓	NA	NA
		Predazzo	✓	✓	✓	✓	✓	NA	NA
	L. simulans	Tuscany	NA	NA	✓	✓	✓	✓	✓
		Tuscany.2	NA	×	✓	NA	NA	✓	✓

Figure 6. Seven population experiment. Matrix displaying F_2 progeny production success for each reciprocal combination that produced F_2 offspring. Dark coloured cells with NAs represent those combinations where no F_1 generation was produced. Light shaded NA cells represent those combinations where F_1 progeny were produced but insufficient numbers survived to test reliably for F_2 offspring production. Only one combination (Tuscany2 male crossed with Morga female) produced no F_2 generation with sufficient numbers of F_1 offspring to test this (3 males and 3 females), however this was derived from only one replicate.

F_2 offspring production

Within species crosses showed no restrictions in the ability of populations to interbreed, and produce fertile hybrids, as F_2 generations were produced in each case (Figure 6). However, the presence of an F_2 generation for some inter-specific crosses does show that *L .equestris* and *L. simulans* are not completely reproductively isolated. Crosses between *L. equestris* males and *L. simulans* females did not produce any F_1 offspring, and indeed only one of these pairings mated in the first instance (Figure 3). However, the reciprocal crosses between male *L. simulans* and female *L. equestris*, were able to mate (Figure 1 and 3), produce an F_1 generation (Figure 4) and a subsequent F_2 generation in some cases (Figure 6). As only a subset of female *L. equestris* and male *L. simulans* pairs produced sufficient numbers of F_1 offspring that developed to adulthood, and could reliably test for F_2 offspring (i.e. at least 3 males and 3 females, or otherwise produced F_2 offspring), I could not determine with certainty whether particular crosses are incompatible. Nevertheless, sexual isolation between *L. equestris* and *L. simulans* would appear to be largely in terms of pre-mating isolation rather than post-mating isolation therefore, and one-directional (i.e. asymmetric pre-mating sexual isolation). Offspring survivorship to adulthood appears low generally amongst *L. equestris*, (Kugelberg 1973c; Kugelberg 1973a), and is likely also true of *L. simulans*. Thus, while the low survivorship of hybrids to test for F_2 generation may indicate reduced fitness of hybrids, it may also reflect low survivorship within these species. Whilst ascertaining the relative fitness of hybrid offspring is interesting, it was not the purpose of this study *per se*. Rather I aimed only to see if such hybrids were produced in the first instance and whether they were reproductively fertile. All told, the data suggest partial pre-mating isolation between *L. equestris and L. simulans* populations that is predominantly one-directional (asymmetric) in nature, whilst no such reproductive barriers appear to be present among populations of *L. equestris*, sampled from across their geographic distribution in the wild.

Discussion

I investigated the extent of reproductive isolation within and between two sister species of seed bug (*L. equestris* and *L. simulans*) that are characterised by sexual conflict over mating (Chapter 4, see also Shuker *et al.* 2006). There was strong pre-mating isolation between *L. equestris* and *L. simulans*, and this was asymmetric: male *L. simulans* were able to mate with female *L. equestris*, but male *L. equestris* were largely unable to mate with female *L. simulans*. I found little evidence for strong post-mating isolation between the two species however, with hybrid F_2 offspring being produced. No sexual isolation was apparent among *L. equestris*, or among *L. simulans*, populations respectively, despite large female mating costs (Chapter 4).

For both of the experiments reported here, (allowing short and prolonged opportunities for mating respectively), heterogeneity in mating was only observed among the populations as a whole due to substantial pre-mating reproductive isolation between *L equestris* and *L. simulans*. This reproductive isolation was asymmetrical in form, with only one *L. equestris* male * *L. simulans* female pair resulting in mating over both experiments, whilst the reciprocal crosses of *L. simulans* male * *L. equestris* female mated more readily, albeit still less than that shown within the two species. However, where mating was achieved in pairs of the first experiment, no difference was found between populations, and species, in the time taken to initiate mating. This suggests that male mating harassment, or persistence, if important for overcoming female resistance and inducing receptivity, is not a function of time, at least for the time-scale studied here (mating scanned every 30 minutes). Asymmetrical mating between *L. equestris* and *L. simulans*, and the production of hybrid F_2 offspring, clearly demonstrates that, given the no-choice mating experiments performed here, the two species can interbreed and produce viable offspring under laboratory conditions, in contrast to one report (Maschler 2002). There is, however, large pre-mating isolation and also likely some post-zygotic isolation between these species, as few F_1 hybrids were produced, and survived to test for an F_2 generation (and hybrid fertility), even where hybrids were produced. The experimental design employed here did not allow specific testing of the relative fitness of hybrid offspring. However, the production of F_2 offspring, from inbreeding F_1 families, demonstrates that both sexes were fertile as hybrids.

Pre-zygotic isolation is expected to be more apparent than post-zygotic isolation, as females in particular, would be under greater selection for pre-zygotic isolation to prevent

wastage of gametes and energy (Dobzhansky 1940; Andersson 1994). Pre-zygotic sexual isolation may act over many systems involving behavioural, physiological and/or morphological characters (Coyne and Orr 1989; Coyne and Orr 1997). Although no overt mating courtship occurs in the two species studied here (Solbreck 1972; Sillén-Tullberg 1981; Tadler *et al.* 1999), morphological differences between the species could explain the pre-mating isolation observed to some extent. Indeed, one of the few characters that distinguish the species, is the morphology of the male parameres (genital claspers, Deckert, 1985; Péricart, 1998, see Chapter 2- Figure 2, and Appendix 1), which are used by males to secure position during mating facilitating successful copulation (Tadler 1999; Tadler *et al.* 1999, and see Chapter 2 for details). The finding of asymmetric reproductive isolation between *L. equestris* and *L. simulans* here questions whether they are indeed 'true' species. However, given that inter-specific mating was low relative to intra-specific mating (and particular considering the no-choice mating design), as well as the low numbers of F_1 hybrids produced in the first instance, and that subsequently survived to test for F_2 offspring, they likely do represent good species in the wild. Other post-zygotic isolating factors, such as the relative fitness of hybrids and conspecific sperm precedence were not studied here however, and close investigation of field populations, where the ranges overlap is needed to resolve the degree of reproductive isolation in the field (e.g. Coyne *et al.* 2002; Coyne and Orr 2004). Asymmetric sexual isolation has been commonly observed in studies of speciation (Arnold *et al.* 1996; Coyne and Orr 1998), such as between populations of the grasshopper, *Podisma sapporensis* (Sugano and Akimoto 2007), among species of *Drosophila* (Markow and Hocutt 1998; Carracedo *et al.* 2000; Coyne *et al.* 2002), and between species of freshwater darter fish (Mendelson 2003a; Mendelson 2003b). Indeed, sexual selection and specifically, divergent sexual antagonistic co-evolution among the populations or species (with differential levels of male vigour and female resistance/preference), appears to be a likely, and generally applicable, explanation for the patterns observed (Markow and Hocutt 1998; Sugano and Akimoto 2007). Thus the observed asymmetry in pre-mating sexual isolation between *L. equestris* and *L. simulans* could reflect sexual conflict and divergent SAC trajectories among groups.

There were no population, or species, differences in mating propensity when paired with partners from their own populations respectively. However, within *L. equestris* crosses there were marginally significant effects of male and female identity on mating, in the four, and seven, population experiments respectively (see Figure 1 and 3). Similarly in the seven population experiment, within mated *L. equestris* crosses, there were significant effects of

both male and female identity on offspring production (Figure 5). This may reflect population differences in aspects of life history (as found in Chapter 4), and/or possibly subtle differences in male persistence (four population experiment, Figure 1), and female resistance to mating, characters (seven population experiment, Figure 3), as found in Chapter 3. The results here support other studies in that despite showing sexual conflict (female mating costs, Chapter 4), there is no evidence that this is associated with population divergence. For example, Gagnon and Turgeon (2011) found that despite significant correlations between morphological traits of males and females, associated with sexual conflict over mating in populations of *Gerris gillettei*, there were no mating asymmetries among allopatric populations, suggesting that sexual conflict was not driving population divergence (Gagnon and Turgeon 2011). Indeed, Styan and coleagues (2008) found that the apparent rapid evolution of reproductive barriers across populations of the polychaete *Galeolaria caespitose*, is unlikely to be driven by arms races derived from sexual conflict. Even in laboratory evolution studies, support for SAC over mating promoting allopatric population divergence is limited (e.g. Wigby and Chapman 2006; Bacigalupe *et al.* 2007; Gay *et al.* 2009; Maklakov *et al.* 2010). Thus, sexual conflict, although possible in the field, may not create an important selective force driving divergence and speciation among *L. equestris* populations. Indeed, these results could perhaps support the contrary theoretical proposition, that male willingness to mate may impede speciation through maintaining gene-flow across populations (Parker and Partridge 1998; Gavrilets and Hayashi 2005).

Chapter 6

General Discussion

General Discussion

Sexual conflict and sexually antagonistic co-evolution have received much attention from researchers of evolutionary biology over the past three decades (Parker 1979; Parker and Partridge 1998; Chapman *et al.* 2003; Arnqvist and Rowe 2005; Lessells 2006; Parker 2006). However, the implications of sexual conflict for evolution are often difficult to predict, and its general role and importance for evolution remains unclear (Gavrilets *et al.* 2001; Gavrilets and Hayashi 2005; Chapman 2006; Lessells 2006). SAC has been invoked far more than shown in natural systems, and sexual conflict may result in many different sexually antagonistic co-evolutionary dynamics including escalating chases, and cyclical dynamics, and thus may act to promote or restrict population diversification, and speciation, in the longer term (Parker and Partridge 1998; Gavrilets and Hayashi 2005). Indeed sexual conflict may not even be expressed in the field as it, by definition, requires opposing selection acting on males and females in any given environment (Chapman 2006).

In this thesis, I explored sexual conflict over mating across multiple populations, of two species of promiscuous *Lygaeus* (Hemiptera: Lygaeidae) seed bugs. The major finding was that although intraspecific populations demonstrate sexual conflict, and show differences in conflict phenotypes (such as the receptivity to mating), as well as other life-history characters, no apparent reproductive divergence has yet occurred among these populations in the wild. These results are discussed in detail within their respective chapters, thus here I briefly comment on the experiments performed and focus on further, unresolved, questions that deserve further study.

Firstly, I asked whether populations found to differ in extent of female mating costs (sexual conflict, Shuker *et al.* 2006), also differed in aspects of their reproductive development and receptivity to mating (Chapter 3). I found that populations did differ in sexual conflict phenotypes in terms of their propensity to mate, and aspects of development also differed between the populations. Quantitative genetic analysis of female receptivity to mating within one of these populations showed that heritabilities of female receptivity were not large. However, it appears that larger experiments will be required to determine the genetic basis, and evolvability, of female receptivity within *Lygaeus equestris*, and is discussed in Chapter 3.

In Chapter 4, I extended a previous study (Shuker *et al.* 2006), to explore the magnitude of sexual conflict (in terms of female mating costs) across multiple populations of *L. equestris*, derived from across its geographic distribution, as well as between *L. equestris* and its closely related sister species *L. simulans*. Female mating costs from multiple mating were large among the populations and species, in line with previous studies (Shuker *et al.* 2006), but depended on the fitness proxy considered. The magnitude of female mating costs were not found to differ across populations however, suggesting that the populations may not differ in the extent of sexual conflict experienced. These populations also differed in aspects of life history, however no obvious patterns between sexual conflict and life histories emerged. In order to investigate how sexual conflict maps onto life-history variation within species, many more populations would need to be studied concurrently.

In Chapter 5, I conducted no-choice mating experiments within and between the populations of *Lygaeus equestris* and *L. simulans* used in the previous chapters, to explore pre- and post-mating reproductive divergence amongst the populations. These reciprocal experiments revealed strong pre-mating reproductive divergence between the two species. However, reproductive isolation between the species was not complete as shown by significantly asymmetric pre-mating isolation. The production of hybrid F_2 offspring showed that post-mating pre- and post-zygotic reproductive isolation, if present, is also not complete between the groups. However, the relatively low numbers of hybrid offspring produced, and considering the no-choice mating design, suggests that they probably do represent "good species" in the field, as multiple isolating mechanisms often occur together to compound overall reproductive isolation (Coyne and Orr 1998; Coyne and Orr 2004). Although hybrids were fertile, post-zygotic isolating mechanisms such as conspecific sperm precedence, and reduced hybrid viability or fitness were not tested here, and may be important (e.g. in sunfish, Immler *et al.* 2011, see also Coyne and Orr 2004). Within *L. equestris*, there was no evidence of reproductive divergence among populations derived from across its geographic distribution, (either within, or between, the regional pairs), yielding no evidence that sexual conflict (and different levels thereof) has led to population divergence.

Further Questions

Receptivity to mating

In Chapter 3 I looked at receptivity to first mating. Studies of sexual conflict over mating in terms of mating rate typically focus on female re-mating characteristics as part of studies on the evolution of polyandry (Arnqvist and Nilsson 2000; Simmons 2005). Chiefly, these include the latency, or time taken, to re-mate, and the likelihood of re-mating (Arnqvist and Nilsson 2000; Simmons 2005). This is due to the need for females to mate at least once in order to gain any fitness. However, although we may expect female receptivity to mating to be plastic, depending on, for example, female ontogeny, condition, and the suitability of mates (Ringo 1996; Wedell 2005; Shamble *et al.* 2009), there may also be fixed, underlying receptivity levels for females, with a certain amount of plasticity (Ringo 1996; Shuker and Day 2001; Dunn *et al.* 2002). As such, testing whether primary receptivity can be informative of subsequent receptivity in *Lygaeus* may prove valuable for sexual conflict over mating whereby female receptivity could potentially then be used as a surrogate for sexual conflict (Shuker and Day 2001). Additionally, reproductive development may also be under sexually antagonistic selection and studies of primary receptivity along with reproductive development may elucidate such patterns. Indeed, the timing of first mating will be important in terms of when animals enter the mating pool and so affect sexual conflict (Blanckenhorn *et al.* 2007).

Sexual conflict phenotype and the extent of conflict

An important question more generally is whether the sexual conflict phenotypes measured (e.g. degree of armament, receptivity to mating, and female mating costs) can reliably inform us about the extent of conflict experienced in the field. Sexual conflict relies on the relative difference between the trait optima of the sexes and, whilst this has long been understood, few studies isolate these as it requires much information on the cost, and benefits, of the traits in both sexes (e.g. Vahed 2007; Fricke *et al.* 2009).

This highlights a potentially important limitation of my studies here. Although both species studied here are highly polygamous (Solbreck *et al.* 1989; Tadler *et al.* 1999; Shuker *et al.* 2006) mating often in the wild (over 60% of individuals mating, Solbreck 1972), and in the lab (females mating over 40 times in their lifetime, Kugelberg 1973b), the actual degree

of polyandry exhibited in the wild is unclear. Thus, the mating rates achieved in the laboratory (where females cannot escape) may over-estimate those typical in the field. However, although the use of partitions to allow females spatial refuge, away from males, within containers is precluded in *L. equestris* and *L. simulans*, due to females being larger than males, temporal refuges from males were provided to better represent natural conditions. Therefore, it remains to be tested whether the laboratory mating rates enforced here, by manipulating sex ratio, inflate the costs of mating above, and beyond, those experienced in the wild, and consequently the level of conflict attributed to them. Nevertheless, the results show that given the laboratory conditions, the costs of mating to females can be large, and that there is thus great potential for conflict in the field. Microsatellite paternity analysis of offspring of wild females throughout the breeding season would provide conservative estimates of polyandry (e.g. Bretman and Tregenza 2005), and analysis of sperm in the female reproductive tract may also shed light upon issues of cryptic female choice and sperm use. Ultimately however, traditional mark, release, and recapture methods over the breeding season, recording the number of partners mated by individuals may be the most accurate way of gaining insight into the mating rate of males and females in the wild, although such studies are time and labour intensive.

Male mating costs

Information on the costs to mating for males is also lacking in this system. It is often assumed that male fitness should increase monotonically with mating rate (Bateman 1948), but males cannot mate indefinitely, and sperm deficiency and nutrition will affect the costs, and so marginal benefits, of further mating for males (e.g. Vahed 2007). Thus, mating may not always be selected for in males, although preventing the female from mating another male will be. In bush-crickets, for instance, Vahed (2007) showed that ejaculate and nuptial gift size both correlated positively with latency to re-mate, suggesting there to be a general trade-off between current reproduction (paternity insurance) and mating rate in males, across these species. To date, surprisingly little work has been carried out on male costs of reproduction (but see Wedell *et al.* 2002a; Kotiaho and Simmons 2003; Vahed 2007; Wedell 2010), and should be addressed, as this is vital to fully understand the difference in trait optima for males and females, and so the degree of conflict expected (Arnqvist and Rowe 2005; Fricke *et al.* 2009). Preliminary analysis in *L. equestris* suggests that mating is indeed costly for males (Shuker et al, unpublished data).

Economic studies of the fitness consequences of increasing mating rates, for both males and females, are needed to determine the degree of conflict between the sexes over mating, and should be assessed to determine how apparent, and general, sexual conflict over mating is in nature (Fricke *et al.* 2009). Unfortunately, the prolonged mating durations exhibited by *Lygaeus* bugs (Sillén-Tullberg 1981) make direct manipulation of mating rates unfeasible in this system. Other systems, with low mating durations, would be more amenable to economic studies of mating rates therefore. However, in *Lygaeus*, males may prolong mating to restrict the opportunity for females to mate with other males (copulatory mate guarding), giving rise to conflict over mating duration (conflict over male defensive sperm competition, Gavrilets and Hayashi 2006). Prolonged mating may be detrimental to females, not only in terms of the direct effect of being unable to mate with potentially better or more suitable mates, but also in terms of pleiotropic effects, such as restricting movement, and impeding oviposition and defecation. Intuitively, males will be under selection not to hinder oviposition of eggs they have fertilised, and signalling mechanisms such as female rocking (and even kicking) behaviours during mating which are common in insects (Walker 1979; Crudgington and Siva-Jothy 2000; Arnqvist and Rowe 2005), may have originated to this end, if they are selected for in both the sexes. However once established, females could feasibly elaborate and intensify such rocking, and kicking, behaviours to their benefit, and to the detriment of males (in sperm competition) leading to SAC analogous to manipulative male traits in initiating mating (Arnqvist and Rowe 2005). Indeed, as stated in Chapter 2, gonad morphology is complex in this system, and in Lygaeidae generally (Bonhag and Wick 1953; Tadler *et al.* 1999; Gschwentner and Tadler 2000; Micholitsch *et al.* 2000), suggesting there may well be fruitful avenues of research in investigating cryptic female choice and reproductive morphological and physiological properties of females. Indeed, fertilisation failures are common in *L. simulans*, and may reflect cryptic female choice over fertilisation (Tadler *et al.* 1999; Gschwentner and Tadler 2000; Micholitsch *et al.* 2000). Furthermore, intriguing recent work on female reproductive morphology of three species of Lygaeidae, suggest the evolution of different structures for sperm insemination and sperm transfer, for fertilisation (Chiang 2010). Thus, economic studies of the costs and benefits of mating duration and female rocking and kicking phenotypes may elucidate further the extent of conflict in these systems.

Sexual conflict and persistent males

Although initially there was much excitement about the huge potential for sexual conflict in driving speciation, recently this has been questioned and the ability of conflict to prevent or restrict divergence has been highlighted, as conflict may be expected to produce generally persistent males and indiscriminate males (Parker and Partridge 1998; Gavrilets and Hayashi 2005; Gagnon and Turgeon 2011). Thus, an interesting avenue of research presents itself in investigating the propensity of sexual conflict to induce reproductive interference among closely related sympatric or parapatric populations, and preventing or hindering adaptive evolution of populations, and this is currently being investigated in the Shuker laboratory.

Condition dependence of sexual conflict

Sexual conflict is defined by opposing selection on males and females, however selection in the field is expected to be dynamic, as ecological and environmental conditions constantly fluctuate (Wedell *et al.* 2002b; Härdling and Kaitala 2005; Kokko and Rankin 2006; Fricke *et al.* 2009). Thus, "conflict" may not always arise in the field, or may only occur in sporadic episodes (Wedell *et al.* 2002b; Magellan and Magurran 2006; Edward and Gilburn 2007; Darden and Croft 2008; Eldakar *et al.* 2010a; Maklakov *et al.* 2010). Therefore, longitudinal studies, measuring temporal variation in the cost of mating, or other conflict phenotypes, in the field would be hugely beneficial to infer when selection on males and females diverge (i.e. when conflict is apparent, Jann *et al.* 2000; Haddrill *et al.* 2007), and how long they must remain divergent for, for SAC to result. Furthermore, longitudinal studies of conflict among replicate populations may shed light on the propensity of sexually antagonistic coevolution to operate along divergent trajectories across populations. They would also be beneficial in highlighting the response of the sexes to conditions of sexual conflict and how this may feed back on conflict (e.g. sexual segregation in guppies, Magellan and Magurran 2006). Although there has been considerable work on experimental evolution studies exploring SAC, there is scope for adding more ecological reality. For instance, laboratory studies could facilitate longitudinal studies by examining sexual conflict and antagonistic coevolution over a range of environmental conditions, and indeed under temporally fluctuating conditions.

Concluding Remarks

Evidence of the context dependence of sexual conflict is mounting from both laboratory and field experiments (Wedell *et al.* 2002b; Croft *et al.* 2006; Kokko and Rankin 2006; Magellan and Magurran 2006; Darden and Croft 2008; Eldakar *et al.* 2009a; Reinhardt *et al.* 2009; Edward *et al.* 2010; Eldakar *et al.* 2010a; Eldakar *et al.* 2010b). This suggests that environmental and/or ecological conditions are crucial for the expression of sexual conflict, and thus its importance for evolution (Fricke *et al.* 2009). The work described in this thesis highlights the fact that female mating costs can be large. However, the extent of costs can differ depending on the fitness measure used, and this highlights the importance of careful consideration of the fitness components measured for interpreting empirical results. While this point has been made before (e.g. Arnqvist & Nilsson 2000), the data presented here provide a particularly clear example. However, despite displaying substantial sexual conflict among populations, there was no evidence of intraspecific reproductive isolation for *Lygaeus equestris*. The challenge now will be to explore sexual conflict in longitudinal, time-series, studies of natural populations to describe how patterns of sexual conflict and the dynamics of antagonistic evolution manifest over time in natural environmen

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Appendix 1

Population identification:

L. equestris and *L. simulans*

Appendix 1

Morphological comparisons: *L. equestris* and *L. simulans*

Morphological comparisons of all populations of *Lygaeus* experimented upon in this thesis. Differentiation of *L. equestris* from *L. simulans* is determined by morphological characteristics of the antennae base and the male parameres (see Chapter 2, and below). The Dolomites and Leeds populations of *L. equestris* were used in Chapter 3. Chapter 4 uses Swedish *L. equestris* populations (Geta and Morga), Italian *L. equestris* populations (Ledro and Predazzo), and Italian *L. simulans* populations (Tuscany and Tuscany.2 that were derived from same area). Chapter 5 used all the populations except the laboratory adapted population Leeds, *L. equestris* population derived from Sicily.

(A) Geta

(B) Morga

(C) Ledro

(D) Predazzo

(E) Tuscany

(F) Tuscany.2

(G) Dolomites

(H) Leeds

Figure 1. Images of the head and antennae base of adults from each of the respective populations used in this thesis. A – D are recently caught field populations of *L. equestris*. E and F are field caught populations of *L. simulans*. G and H are laboratory populations of *L. equestris*.

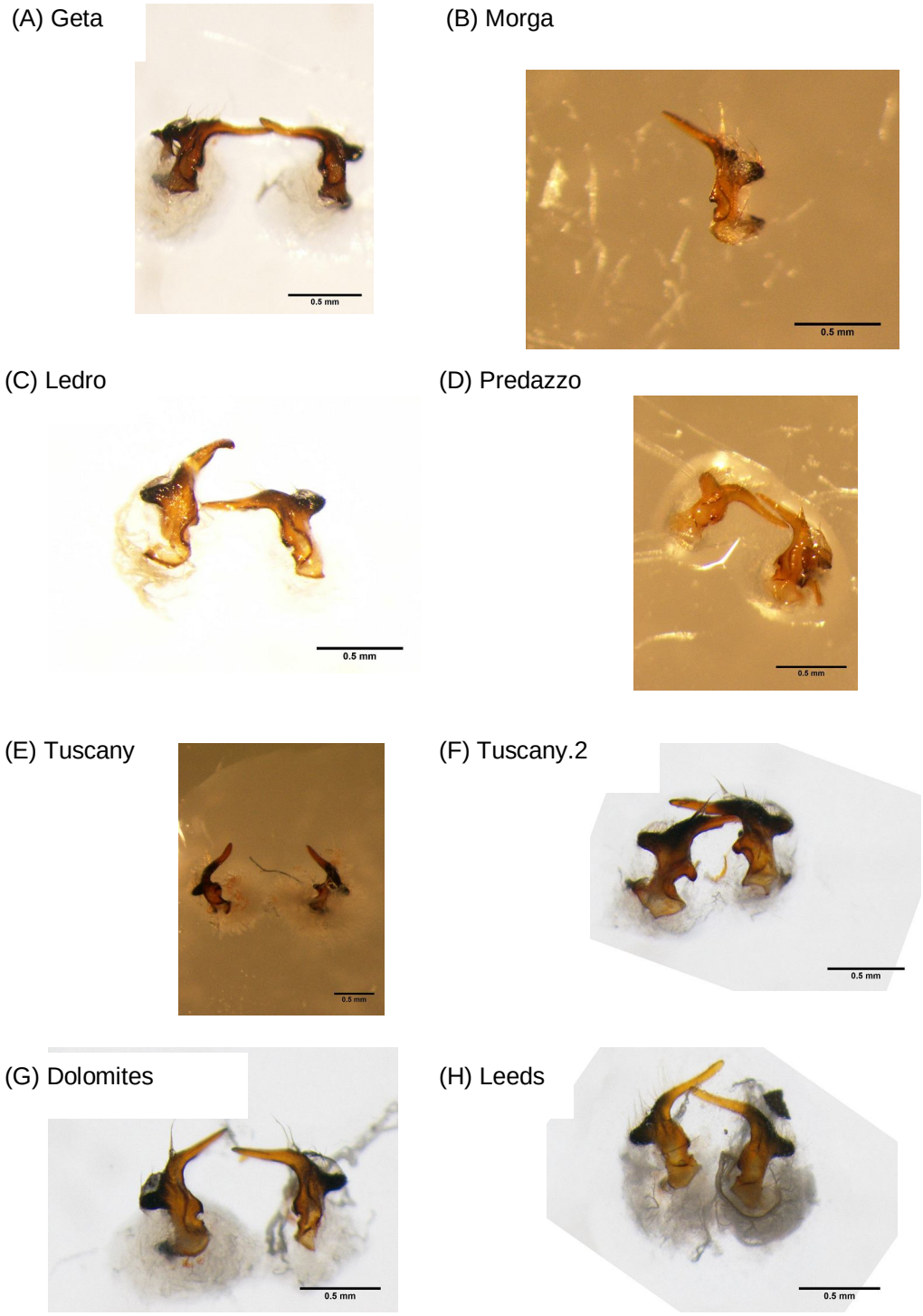

Figure 2. Images of the parameres (genital claspers) of male adults from each of the respective populations. A – D; recently caught field populations of *L. equestris*. E and F; field caught populations of *L. simulans*. G and H; laboratory populations of *L.equestris*.

Appendix 2

Experimental setup for experiments in Chapter 5

Appendix 2

This section refers to the experiments performed in Chapter 5, where I explored the propensity of males and females from each population to mate (both the four-population and seven-population experiment) and breed (seven-population experiment) in reciprocal cross experimental designs. The figures and tables below, outline the numbers of replicate pairs achieved for each pair combination in the mating and breeding experiments respectively, as well as the raw data in terms of numbers of pairs found mating, and breeding (producing offspring). See Chapter 5 for details of the experimental design and analysis.

Table 1. Contingency tables of the incidence of mating from reciprocally crossed, no-choice, experimental pairs within and between populations for the (A) four population and (B) seven population experiments described in Chapter 5. The marginal frequencies (*N*) are the number of males (along the top) and females (down the side) used from each population (shaded). A total of 100 pairs were setup in the four population experiment (A), 25 for each possible combination in a balanced design. Numbers within the table are the number of pairs observed mating during eight hour mating trials. For the seven population experiment (B), 491 pairs were obtained, and numbers within the table are the number of pairs observed mating out of the number of replicates obtained for each cross (in bold).

A.

				L. equestris			*L. simulans*
			Males	Morga	Geta	Dolomites	Tuscany
		Females	*N*	100	100	100	100
L. equestris		Morga	100	21	23	23	2
		Geta	100	19	21	25	0
		Dolomites	100	19	23	23	8
L. simulans		Tuscany	100	0	0	0	24

B.

				L. equestris					L. simulans	
			Males	Dolomites	Geta	Ledro	Morga	Predazzo	Tuscany	Tuscany.2
		Females	*N*	65	71	76	72	71	65	71
L. equestris		Dolomites	66	7 ***8***	8 ***9***	9 ***9***	9 ***11***	10 ***10***	6 ***9***	1 ***10***
		Geta	72	9 ***9***	6 ***9***	11 ***14***	7 ***10***	7 ***10***	3 ***10***	1 ***10***
		Ledro	73	7 ***10***	10 ***11***	8 ***11***	7 ***10***	10 ***11***	6 ***8***	8 ***12***
		Morga	68	8 ***9***	5 ***10***	11 ***11***	10 ***10***	10 ***10***	4 ***9***	7 ***9***
		Predazzo	75	9 ***9***	9 ***12***	9 ***11***	8 ***11***	8 ***10***	6 ***12***	5 ***10***
L. simulans		Tuscany	70	1 ***10***	0 ***11***	0 ***10***	0 ***10***	0 ***10***	6 ***9***	10 ***10***
		Tuscany.2	67	0 ***10***	0 ***9***	0 ***10***	0 ***10***	0 ***10***	8 ***8***	9 ***10***

			Female																			
			L. equestris														L. simulans					
			Geta			Morga			Dolomites			Ledro			Predazzo			Tuscany			Tuscany.2		
Male	L. equestris	Geta	6	5	1.0	7	6	1.0	8	7	1.0	8	7	1.0	8	8	1.0	NA	NA	NA	0	NA	NA
		Morga	4	3	1.0	8	7	0.9	10	10	1.0	8	8	1.0	6	6	1.0	NA	NA	NA	NA	NA	NA
		Dolomites	7	6	1.0	7	7	1.0	7	7	1.0	6	6	1.0	8	8	1.0	NA	NA	NA	NA	NA	NA
		Ledro	6	5	1.0	6	4	1.0	9	9	1.0	6	6	1.0	8	8	1.0	NA	NA	NA	NA	NA	NA
		Predazzo	4	4	1.0	8	8	1.0	10	10	1.0	6	6	1.0	6	6	1.0	NA	NA	NA	NA	NA	NA
	L.simulans	Tuscany	1	NA	NA	NA	NA	NA	6	4	1.0	5	2	0.5	2	2	0.5	4	3	1.0	7	6	1.0
		Tuscany.2	1	NA	NA	3	1	0.0	2	1	1.0	2	NA	NA	1	NA	NA	7	7	1.0	5	4	1.0

Figure 1. Matrix describing the ability of pairs from the same and different, populations and species to interbreed and produce F_2 progeny from the seven population experiment. For each female population, the 1st column (whit cells) shows the number of replicates that produced F_1 offspring for each combination. The 2nd column (lightly shaded cells) displays the number of replicates obtained with sufficient F_1 generation adults (see methods for details) to test for F_2 offspring (NAs represent those combinations where no replicates were available due to insufficient survival of F_1 progeny, or no F_1 progeny was produced). The 3rd column (dark shaded cells) shows the proportion of replicates (in column 2) that produced F_2 progeny.